NON-CHOLESTEROL STEROLS IN SERUM, LIPOPROTEINS, AND RED CELLS

Effects of Dietary Phytosterols in Familial Hypercholesterolemia

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Academic Dissertation

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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I Ketomäki A, Gylling H, Siimes MA, Vuorio A, Miettinen TA. Squalene and noncholesterol sterols in serum and lipoproteins of children with and without familial hypercholesterolemia. Pediatr Res 2003;53:648-653.

II Ketomäki AM, Gylling H, Antikainen M, Siimes MA, Miettinen TA. Red cell and plasma plant sterols are related during consumption of plant stanol and sterol ester spreads in children with hypercholesterolemia. J Pediatr 2003;142:524-531.

III Ketomäki A, Gylling H, Miettinen TA. Non-cholesterol sterols in serum, lipoproteins, and red cells off and on plant stanol and sterol ester spreads in statin-treated FH subjects. Submitted.

IV Ketomäki A, Gylling H, Miettinen TA. Effects of plant stanol and sterol esters on serum phytosterols in a family with familial hypercholesterolemia including a homozygous subject. J Lab Clin Med 2004;143:255-262.

V Ketomäki A, Gylling H, Miettinen TA. Removal of intravenous Intralipid in patients with familial hypercholesterolemia during inhibition of cholesterol absorption and synthesis. Clin Chim Acta 2004;344:83-93.

ABBREVIATIONS

ABCA1	adenosine triphosphate-binding cassette transporter A1
ABCG5	adenosine triphosphate-binding cassette transporter G5
ABCG8	adenosine triphosphate-binding cassette transporter G8
ACAT	acylcoenzyme A:cholesterol acyltransferase
ANOVA	analysis of variance
apo	apolipoprotein
AUIC	area under incremental curve
CETP	cholesterol ester transfer protein
CHD	coronary heart disease
CM	chylomicron
d	density
FH	familial hypercholesterolemia
FH-Hki	FH-Helsinki
FH-NK	FH-North Karelia
GLC	gas liquid chromatography
HDL	high density lipoprotein
HL	hepatic lipase
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HSBG	heparan sulfate proteoglycans
IDL	intermediate density lipoprotein
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LPL	lipoprotein lipase
LRP	LDL receptor-related receptor
LXR	liver X receptor
MTP	microsomal transfer protein
PCR	polymerase chain reaction
PLTP	phospholipid transfer protein
RLP	remnant-like particle
SR-B1	scavenger receptor class B type 1
TG	triglycerides
TRL	triglyceride-rich lipoprotein
VLDL	very low density lipoprotein

ABSTRACT

Introduction: Serum squalene and non-cholesterol sterols, cholesterol precursor sterols (Δ^8-cholestenol, desmosterol, and lathosterol) and absorption marker sterols (plant sterols and cholestanol) reflect in general cholesterol metabolism, but limited data exist on their distribution in different lipoproteins. Dietary plant stanols and sterols, called phytosterols, can lower serum cholesterol levels by inhibiting cholesterol absorption. In addition, dietary plant stanols decrease and plant sterols increase the serum plant sterol levels, whereas both phytosterol products increase the serum levels of cholesterol precursor sterols. Recent data have suggested that high serum plant sterol levels are associated with coronary heart disease (CHD), indicating that dietary plant sterols increasing their own serum concentrations could be harmful especially in subjects with high risk for CHD, such as patients with familial hypercholesterolemia (FH). Furthermore, statin treatment increases the serum plant sterol levels, but the data from combination therapy of statins and plant sterols are limited. Accordingly, we measured squalene and non-cholesterol sterols in serum, lipoproteins, and red cells of hypercholesterolemic subjects, mainly those with FH, at baseline and during consumption of dietary phytosterol esters off and on statin treatment. In addition, the effects of dietary phytosterols on clearance of postprandial lipoproteins were studied.

Subjects and methods: In Study I, we measured non-cholesterol sterols in serum and different lipoproteins in 18 FH and 29 non-FH children. In Studies II and III, we studied the effects of dietary phytosterols on serum and red cell non-cholesterol sterol levels in 23 hypercholesterolemic children (aged 2-9 years) and in 18 statin-treated FH adults consuming 1.7-2.0 g/day of plant stanols and sterols for five and four weeks, respectively with a cross-over design. We evaluated the non-cholesterol sterol levels in different lipoproteins at baseline and during consumption of dietary phytosterol esters in Study III as well as in the FH family study (Study IV) including one homozygous FH subject. Study V was a substudy of Study III and consisted of five statin-treated FH subjects, in whom we evaluated the effects of dietary phytosterols on the clearance of postprandial lipids using intravenous Intralipid-squalene fat emulsion.

Results: Serum concentrations of non-cholesterol sterols were higher in FH than in non-FH children, whereas the ratios of non-cholesterol sterols to cholesterol in serum and lipoproteins were similar in the two groups. Low density lipoprotein (LDL) is responsible for the transport of the major part of the non-cholesterol sterols similarly as it transports cholesterol. The ratios of cholesterol precursors to cholesterol were higher in very low density lipoproteins (VLDL) than in serum, whereas those of plant sterols and cholestanol were mostly higher in high density lipoprotein (HDL) than in serum in both groups of children, in statin-treated FH subjects, and in the homozygous FH subject, also during consumption of dietary phytosterols. Serum total and LDL cholesterol were decreased by dietary phytosterol esters. In all intervention studies serum plant sterol concentrations and ratios to cholesterol were increased by up to 75% by plant sterol esters and decreased by up to 40% by plant

stanol esters. The increase of serum plant sterols by plant sterol esters was highest in those subjects with the highest baseline serum plant sterol levels, in hypercholesterolemic children and in statin-treated FH, whereas the highest serum baseline plant sterol ratios predicted the greatest decrease in serum plant sterols by the consumption of plant stanol ester spread. In particular, the changes occurring in the levels of plant sterols in red cells were in close correlation with the respective changes in serum. The ratios of serum cholesterol precursor sterols to cholesterol which can be viewed as a marker of increased cholesterol synthesis were increased in hypercholesterolemic children and also in statin-treated FH subjects. The changes of non-cholesterol sterols in lipoproteins were similar to those seen in serum. The consumption of dietary phytosterols unexpectedly impaired the clearance of Intralipid squalene, and to a lesser extent of plant sterols, especially in chylomicrons.

Conclusions: Despite differences in the concentrations of non-cholesterol sterols in serum and lipoproteins, the ratios of non-cholesterol sterols to cholesterol in serum and lipoproteins were similar in FH and non-FH children. The different values of non-cholesterol sterols in lipoproteins compared with serum values suggest that even though the serum ratios of non-cholesterol sterols do provide some kind of picture of cholesterol metabolism in general, the ratios of non-cholesterol sterols in different lipoproteins may reveal more precisely the changes in cholesterol synthesis and absorption. The serum, lipoprotein, and red cell plant sterol levels were increased by plant sterol ester spread and decreased by plant stanol ester spread in all of the intervention studies, especially in those statin-treated FH subjects who had high baseline serum plant sterol levels. According to the literature, an increase in the serum plant sterol levels could be harmful to the arterial wall. The close correlation between serum and red cell plant sterol does suggest that the change of serum plant sterols is accompanied by respective change in the red cells. Thus, especially in FH subjects who already are at high risk of CHD, the dietary agents such as plant stanol esters, which lower both LDL cholesterol and plant sterol levels in serum are recommended for use as monotherapy and in combination with statins. The reason for impaired clearance of postprandial lipoproteins in statin-treated FH subjects is not clear, but could be related to altered lipoprotein metabolism due to impaired LDL apolipoprotein B receptor and statin therapy.

1. INTRODUCTION

A high concentration of cholesterol in serum, i.e., hypercholesterolemia, is a well-known risk factor for coronary heart disease (CHD). Familial hypercholesterolemia (FH) is an inherited disease leading in the heterozygous form to a two- to three-fold and in the homozygous form to a four- to five-fold increase in serum cholesterol levels compared with normolipidemic subjects. This elevation is accompanied by an increased risk for premature CHD (Goldstein et al. 2001). Early atherosclerotic lesions have been detected even in children with FH (Tonstad et al. 1996). Thus, early treatment with a diet low in saturated fat combined with medications, such as statins and bile acid sequestrants is recommended.

Plant sterols and stanols, in general called phytosterols, are constituents of plants and have chemical structures which resemble cholesterol. Sitosterol and campesterol are the most common naturally occurring plant sterols, whereas their saturated derivatives sitostanol and campestanol are much less abundant in plant materials. Phytosterols lower serum cholesterol levels by inhibiting cholesterol absorption (Grundy et al. 1969, Heinemann et al. 1991), and since the 1950's they have been used as cholesterol-lowering agents, first in a free form (Pollak and Kritchevsky 1981) and recently mainly as esterified phytosterols (Katan et al. 2003). Plant stanol and sterol esters as monotherapy reduce low density lipoprotein (LDL) cholesterol by ~15% in different study populations including FH subjects (Katan et al. 2003). A similar additional reduction in LDL cholesterol has been obtained by adding phytosterols to concurrent statin treatment. In addition, during consumption of plant stanol esters, the clearance of postprandial lipoproteins was improved in normolipidemic subjects (Relas et al. 2000), probably by an upregulation of the LDL apolipoprotein (apo) B receptors (Plat and Mensink 2002a). However, little is known about the effects of phytosterols on postprandial lipoproteins in FH.

Dietary plant sterols, but not plant stanols, increase the concentrations of serum plant sterols (Vanhanen and Miettinen 1992b, Hallikainen et al. 2000), since ~5-10% of the plant sterols are absorbed from the intestine (Heinemann et al. 1993). In fact, even low amounts of dietary plant sterols consumed in their free form by children increased the serum plant sterol levels up to ~9 mg/dl (Mellies et al. 1976a). However, nothing is known about the effects of esterified plant sterols on serum plant sterol values in children. In addition to dietary plant sterols, statin treatment increases the serum plant sterol levels (Vanhanen et al. 1992, Miettinen et al. 2000a). Combination of plant sterols to statin treatment further increases the serum plant sterol levels (Neil et al. 2001), while plant stanols inhibit the statin-induced increase in serum plant sterols (Vuorio et al. 2000).

In phytosterolemia, high serum concentrations of plant sterols (~20-93 mg/dl) put subjects at risk of suffering premature CHD (Björkhem et al. 2001). In addition, in non-phytosterolemic subjects, indirect evidence exists for an association between the increased levels of serum plant sterols and the risk of CHD (Glueck et al. 1991, Assmann et al. 2003). Further, serum plant sterols can be incorporated into red blood cell membranes (Salen et al. 1970), and increased levels of plant sterols in red cells

may increase their tendency to undergo hemolysis and the cells have decreased deformability (Bruckdorfer et al. 1969, Björkhem et al. 2001). Thus, these factors raise concerns about whether increased serum and red cell plant sterol levels may have harmful effects, especially in FH subjects with increased risk for CHD.

In addition to cholesterol and plant sterols, serum contains small amounts of other non-cholesterol sterols and squalene. Of these, serum ratios of cholesterol precursor sterols to cholesterol reflect cholesterol synthesis, while those of plant sterols and cholestanol reflect cholesterol absorption (Miettinen et al. 1989, Miettinen et al. 1990b). Non-cholesterol sterols and squalene are transported in lipoproteins, but only limited data exist on their distribution in different lipoproteins, especially in FH and during consumption of phytosterols. It was speculated that perhaps some additional information on cholesterol metabolism could be obtained by analyzing the squalene and non-cholesterol sterol levels in different lipoproteins.

Therefore, the present study concentrated on serum, lipoprotein, and red cell levels of cholesterol, squalene, and non-cholesterol sterols in normolipidemic children and in hypercholesterolemic subjects, including children with FH, adults with heterozygous FH with or without statin treatment, and in one homozygous FH subject. In particular, we wanted to evaluate the effects of plant stanol and sterol esters on serum, lipoprotein, and red cell plant sterol values in these atherosclerosis-prone subjects. In addition, the effects of plant stanol and sterol esters on postprandial lipid clearance measured with an intravenously administered fat emulsion were evaluated.

2. REVIEW OF THE LITERATURE

2.1. Overview of lipoprotein metabolism

In addition to cholesterol, serum contains small amounts of squalene and non-cholesterol sterols such as the cholesterol precursors Δ^8-cholestenol, desmosterol, and lathosterol, and the plant sterols (mainly campesterol and sitosterol) and cholestanol. Since these lipids along with phospholipids and triglycerides (TG) are water-insoluble, they are transported by lipoproteins in human blood. Lipoproteins carry the lipids between the site of their absorption i.e., small intestine, the liver, and extrahepatic tissues. Lipoprotein particles contain a hydrophobic core of TG and cholesterol esters, and a hydrophilic surface of free cholesterol, phospholipids, and apolipoproteins. Plasma lipoproteins are classified based on their density into five classes: chylomicrons (CM, d < 0.94 g/ml), very low density lipoprotein (VLDL, d = 0.94-1.006 g/ml), intermediate density lipoprotein (IDL, d = 1.006-1.019 g/ml), LDL (d=1.019-1.063 g/ml), and high density lipoprotein (HDL, d = 1.063-1.210 g/ml). Due to their high TG content, CM and VLDL are called triglyceride-rich lipoproteins (TRL). The transport of different lipoproteins is summarized in the following text and in Figure 1. This summary is mainly based on references by Gotto (1986), Bruce et al. (1998) and Havel and Kane (2001).

CM, which have a particle diameter of 75-1200 nm, are the largest lipoproteins. They are secreted by the enterocyte. ApoB-48, synthesized by the enterocyte, is the primary structural protein of CM, but CM contain also small amounts of apoA-I and apoA-II synthesized by the intestine. In addition, after secretion of CM, apoC-I-III and apoE are acquired by transfer from HDL. After absorption, TG, cholesterol, and other sterols are packed in CM and are transported via lymph to blood where most of the TG of the CM are hydrolyzed by lipoprotein lipase (LPL) on the surface of capillary endothelial cells to form CM remnants. Concomitantly, apoA, most of the apoC, and phospholipids are transferred to HDL by phospholipid transfer protein (PLTP). The remnant particles are taken up by the liver via several mechanisms that will be described in detail later. The sterols are either stored as esters, excreted into bile as such, or as acidic derivatives, or they can be released into circulation in VLDL.

VLDL particles (30-80 nm) are synthesized in the liver and contain apoB-100 as the main structural protein. In addition, VLDL contains also apoC-I-III and apoE. VLDL particles transport not only endogenously synthesized sterols but also exogenously derived TG and sterols that are not excreted in bile. The release from the liver is followed by hydrolysis of TG by LPL similarly although slower than with CM, leading to the formation of VLDL remnant particles. As hydrolysis proceeds, phospholipids and most of the apoC are transferred to HDL by PLTP followed by transformation of the remnant particles into IDL particles (25-35 nm) and further by lipolysis by hepatic lipase (HL) into LDL. In humans, about 50% of VLDL is converted to LDL. Part of the remnants and IDL particles are taken up by the liver receptors, mostly via apoE.

LDL, with a particle diameter of 18-25 nm, is a major carrier of cholesterol in plasma. It contains only one molecule of apoB-100 as a structural protein. Most of the LDL particles are taken up into the liver by receptor-mediated pathways by recognition of apoB-100, but peripheral tissues that also contain LDL receptors can take up an appreciable portion of LDL.

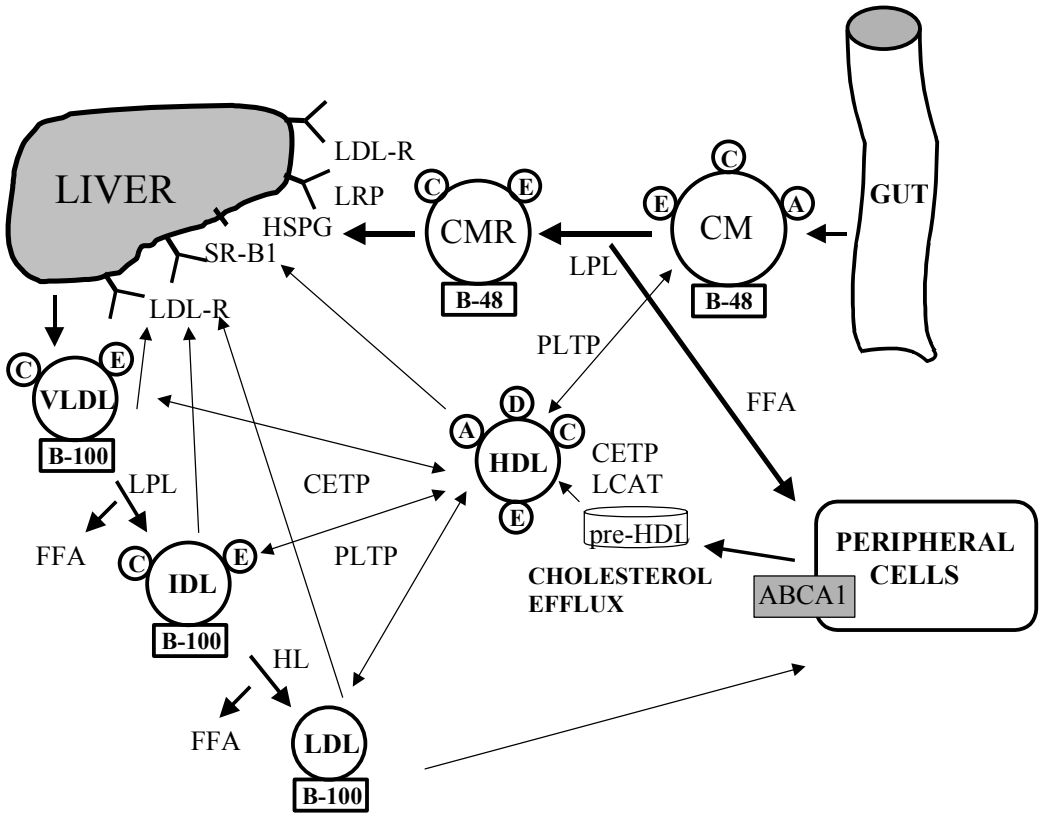

Figure 1. Overview of lipoprotein metabolism.
A, apolipoprotein A; ABCA1, adenosine triphosphate-binding cassette transporter A1; B-48, apolipoprotein B-48; B-100, apolipoprotein B-100; C, apolipoprotein C; CETP, cholesterol ester transfer protein; CM, chylomicron; CMR, chylomicron remnant; E, apolipoprotein E; FFA, free fatty acids; HDL, high density lipoprotein; HL, hepatic lipase; HSPG, heparan sulfate proteoglycans; IDL, intermediate density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; LDL-R, low density lipoprotein receptor; LPL, lipoprotein lipase; LRP, low density lipoprotein receptor-related protein; PLTP, phospholipid transfer protein; SR-B1, scavenger receptor class B type 1

HDL particle (5-12 nm) contains apoA-I, apoA-II, and apoA-IV, which are synthesized by the liver and intestine and are carried to HDL either as free apos or by PLTP from CM. HDL contains also apoC-I-III and apoE mainly transported from CM, VLDL, and their remnants by PLTP. HDL contains also small apoD originating from many tissues. Discoidal nascent pre-β-HDL contains apoA-I, phospholipids, and

cholesterol but not cholesterol esters. This nascent HDL carries lecithin:cholesterol acyltransferase (LCAT), which is capable of esterifying free cholesterol. The substrate for LCAT is derived from plasma membranes, where the adenosine triphosphate-binding cassette transporter A1 (ABCA1) facilitates the transport of free cholesterol to nascent HDL. As the cholesterol is esterified by LCAT, pre-β-HDL is matured to HDL. Cholesterol ester transfer protein (CETP) facilitates the transport of cholesterol esters in exchange for TG to apoB containing lipoproteins, which are taken up by the liver as explained above. HDL cholesterol can also be selectively taken up by the liver either via scavenger receptor class B type 1 (SR-B1) or in the case of a small part of apoE containing HDL via the LDL apoB receptor. TG derived from apoB containing lipoproteins in HDL are hydrolyzed by HL and smaller discoidal pre-β-HDL are regenerated to restart the cycle. Thus, HDL accompanied by CETP mediates the reverse cholesterol transport from the peripheral tissues to the liver to be eventually delivered into the bile.

2.2. Phytosterols - structure, function, and sources

Plant sterols and stanols, in general called phytosterols, resemble structurally mammalian cholesterol such that they are made of a tetracyclic cyclopent[a]phenanthrene ring with a 3β-hydroxyl group and an alkyl side chain at the C-17 carbon atom (Figure 2) (Piironen et al. 2000a). The most common phytosterols are 4-desmethyl sterols that lack methyl groups at C-4 position. They differ structurally from cholesterol in their side-chain substitution, in that campesterol possesses an additional methyl and sitosterol has an ethyl group at C-24 position. Some plant sterols, e.g., stigmasterol have a Δ^{22}-double bond in their side chain. The position of double bond at B ring can also be changed, categorizing the plant sterols as Δ^{5}-sterols, Δ^{7}-sterols, or $\Delta^{5,7}$-sterols. Saturation of the ring structure at carbon 5 leads to formation of plant stanols, such as campestanol and sitostanol. The 3β-hydroxyl group may be esterified by a fatty acid or a phenolic acid to produce steryl esters (sterol esters), or it may be linked to a carbohydrate to form steryl glycosides or acyl steryl glycosides.

Phytosterols are synthesized, as cholesterol, from acetyl coenzyme-A via squalene (Piironen et al. 2000a). They occur as membrane constituents in plants, where they regulate the fluidity and permeability of membranes (Hartmann 1998). They also serve as precursors for plant steroid hormones, the brassinosteroids (Clouse 2000). Phytosterol esters are located intracellularly and act as a storage form of the sterols.

Human cells are not able to synthesize phytosterols (Salen et al. 1970). Thus, the only source of these sterols for humans is the diet. Various plant and marine materials include over 250 different phytosterols and related compounds (Akihisa et al. 1991), of which sitosterol (65%) is the most common, followed by campesterol (32%) and stigmasterol (3%) (Weihrauch and Gardner 1978, Björkhem et al. 2001). The intake of phytosterols varies among different populations according to the food products being consumed, but the average daily Western diet is reported to contain 150-300 mg of these sterols (de Vries et al. 1997, Phillips et al. 1999, Björkhem et al. 2001). However, vegetarians may consume up to 1 g of phytosterols each day (Vuoristo and Miettinen 1994).

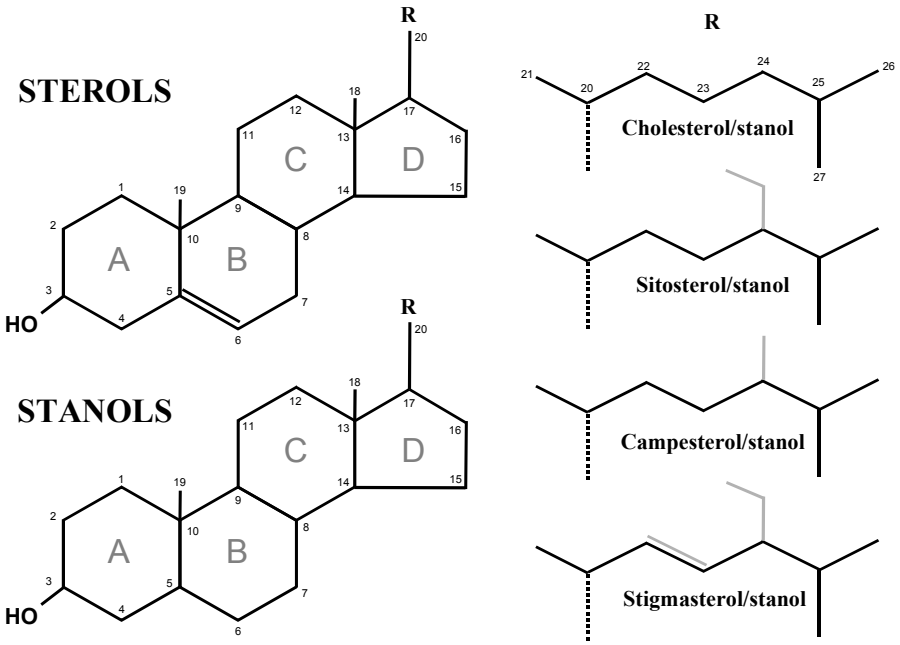

Figure 2. Structure of most common plant sterols and stanols.

The major part of the dietary phytosterols are plant sterols, whereas the intake of saturated plant stanols is about 10% of the intake of plant sterols (Czubayko et al. 1991). Vegetable oils and fats are the richest sources of plant sterols in diet (Weihrauch and Gardner 1978, Piironen et al. 2000a). In addition, cereals, nuts, seeds, vegetables, fruits, and berries contain different amounts of plant sterols. On average, vegetable oils contain 100 to 500 mg of plant sterols per 100 g oil, while corn, rapeseed, rice bran, and wheat germ oils contain higher amounts of plant sterols. In Finland, the most common oil used in margarines, rapeseed oil, contains 700 mg plant sterol per 100 g oil (Piironen et al. 2000b). Accordingly, the soft margarines mostly used in Finland with fat contents from 40% to 80%, contain 130-540 mg/100g of plant sterols. From the cereal products mostly used in Finland, rye is the best source of plant sterols (~90 mg/100g of fresh weight) (Piironen et al. 2000b) and, accordingly, is an important source of plant sterols due to its high daily consumption. In addition, rye and wheat also contain plant stanols in small amounts (~20 mg/100g). Nuts and seeds are rich in plant sterols, the content ranging from 22 mg/100g (chestnut) to 714 mg/100g (sesame seed) (Weihrauch and Gardner 1978). Vegetables, fruits, and berries are poor sources of plant sterols (~5-40 mg/100 g of fresh weight) because of their high water content.

2.3. Sterol absorption

In addition to 150-300 mg of phytosterols, the diet in Western countries contains daily approximately 300-500 mg of cholesterol (Grundy 1983), which makes a miniscale (i.e., about 0.5%) contribution to the total dietary fat, the major part of

dietary fat being TG (95%) (Jones and Kubow 1999). In addition to dietary sterols, the intestinal sterols consist of biliary sterols (e.g., cholesterol 800-1200 mg/day) and of variable amounts of cholesterol synthesized by the intestinal cells (Grundy 1983, Wilson and Rudel 1994). All of the biliary sterols are in a free form, but the proportion of esterified sterols obtained from the diet varies greatly, ranging from 1% up to 73% for cholesterol (Vuoristo and Miettinen 2000), and from ~10% up to 80% for phytosterols (Piironen et al. 2000a). Thus, the bulk of intestinal sterols consists of up to 1.5-2.0 g of free and esterified sterols from endogenous and exogenous sources.

On average, 40-60% of the dietary cholesterol is absorbed (Grundy 1983, Wilson and Rudel 1994), whereas despite the structural similarity, the intestinal absorption of phytosterols is much less. Thus, in humans, when measured with different techniques, the absorption of sitosterol and stigmasterol has been found to be ~5% (Gould et al. 1969, Salen et al. 1970, Heinemann et al. 1993), while the absorption of campesterol is higher (~10-16%) (Heinemann et al. 1993, Lütjohann et al. 1995). Saturation of the Δ^5-double bond further decreases the absorption leading to absorption portions of ~2% for plant stanols (Miettinen et al. 2000b, Miettinen et al. 2000c), even though up to 13% absorption of campestanol has also been observed (Heinemann et al. 1993). Recently, Ostlund et al. (2002) reported extremely low absorption rates for both plant sterols (0.5-1.9%) and stanols (0.04-0.16%) by using a dual stable isotopic tracer technique. These controversial results suggest that the reported absorption rates are dependent on the method used, or may be related to the small sample sizes employed (Heinemann et al. 1993). Generally, the absorption of different sterols decreases with an increased length of side chain in C-24 (Bhattacharyya 1981, Child and Kuksis 1983, Heinemann et al. 1993).

2.3.1. Absorption from intestinal lumen to enterocyte

While the ingested fat is mostly water insoluble, the basic step in fat absorption is conversion of this hydrophobic oil to hydrophilic compounds that can be efficiently absorbed from the intestine. After emulsification and initial hydrolysis by lingual and gastric lipase, the hydrolysis of TG, phospholipids, and esterified sterols takes place in the small intestine principally by pancreatic lipases activated by colipase (Borgström 1975), cholesterol esterase (Vahouny et al. 1964), and phospholipase A2 (van Deenen et al. 1963). However, in addition to free sterols, some absorption of esterified sterols also occurs (Compassi et al. 1995). While the hydrolyzed sterols still have only limited solubility in an aqueous environment, bile acids are needed to form micelles (Siperstein et al. 1952, Swell et al. 1958) to assist the sterols to diffuse across the unstirred water layer to reach the mucosal cell membrane (Westergaard and Dietschy 1976). The micellar solubility of phytosterols has been shown to be poorer than that of cholesterol (Slota et al. 1983, Armstrong and Carey 1987), thus partly explaining the low absorption rate of the phytosterols.

The sterol absorption from the micelles occurs at the brush border membrane of the enterocyte. According to recent data, the cellular uptake is an active event mediated by a transport protein at the brush border membrane (Thurnhofer and Hauser 1990, Thurnhofer et al. 1991, Detmers et al. 2000). Two proteins, pancreatic esterase (Lopez-Candales et al. 1993) and SR-B1 (Hauser et al. 1998) have been postulated as

candidate transport proteins, but animal studies evaluating their role in sterol absorption has not been able to prove this hypothesis (Howles et al. 1996, Mardones et al. 2001, Altmann et al. 2002). Recent studies suggest that at least cholesterol uptake is mediated by a complex of several proteins (Kramer et al. 2003), and it has been postulated that Niemann-Pick C1 like 1 protein (Altmann et al. 2004) and a complex of proteins named annexin2 and caveolin (Smart et al. 2004) would have a critical role in the cholesterol absorption process.

The enterocyte has a crucial role in regulating the uptake of sterols. Integral membrane proteins ABC transporters G5 (ABCG5) and G8 (ABCG8) (Berge et al. 2000, Lee et al. 2001) at the apical surface of the enterocyte pump variable amounts of sterols back into intestinal lumen, thereby regulating the absorption of different sterols. In addition, ABCA1, located at the basolateral surface of the enterocyte has been suggested to have a role in sterol absorption (Repa et al. 2000, McNeish et al. 2000), probably also promoting the efflux of sterols out of the enterocyte. However, the cholesterol absorption efficiency of patients with absent ABCA1 was similar as in non-affected subjects (Schaefer et al. 2001), suggesting that the role of ABCA1 in sterol absorption should be confirmed in humans. Overall, it has been proposed that the efflux from the enterocyte rather than the uptake of intestinal cells is the main process in determining the absorption of different sterols (Igel et al. 2003).

In the endoplasmic reticulum of the enterocytes, cholesterol is re-esterified by acylcoenzyme A:cholesterol acyltransferase (ACAT) (Norum et al. 1979), which is capable of esterifying also sitosterol to some extent (Field and Mathur 1983). However, the limited esterification of sitosterol by ACAT (Field and Mathur 1983, Ikeda et al. 1988a) further contributes to the poorer absorbability of phytosterols. The newly formed sterol esters and TG along with apoB-48 are incorporated into CM this being facilitated by microsomal transfer protein (MTP) (Wetterau et al. 1997, Gordon and Jamil 2000) followed by transport via lymph to bloodstream.

2.3.2. Factors affecting sterol absorption

Cholesterol absorption efficiency exhibits a wide interindividual variation, ranging from 20% to 80% (Sehayek et al. 1998, Bosner et al. 1999). The reasons for this variablity are not completely understood, but several dietary, physiologic, and genetic factors can influence the absorption of cholesterol. Most studies have evaluated the effect of these factors on cholesterol absorption, whereas much less is known about the regulation of phytosterol absorption.

The absorbed mass of dietary cholesterol is increased with the increased intake (Quintão et al. 1971a, Miettinen and Kesäniemi 1989, Sehayek et al. 1998, Ostlund et al. 1999), but the percentual absorption of cholesterol is reported to remain similar (Miettinen and Kesäniemi 1989, Sehayek et al. 1998, Bosner et al. 1999) or may even be decreased (McNamara et al. 1987, Ostlund et al. 1999). In subjects with a high intake of cholesterol, cholesterol absorption was lower in older subjects (75-year-old) than in the younger ones (50-year-old) (Gylling et al. 1994), while no age-related differences were reported in other studies with lower intakes of cholesterol (Bosner et al. 1999). Some conditions with metabolic disturbances, such as high-normal blood

glucose levels in non-diabetic subjects (Strandberg et al. 1996), type 2 diabetes (Simonen et al. 2002), and obesity (Miettinen and Gylling 2000) are associated with low cholesterol absorption efficiency.

In the Finnish population with a high intake of cholesterol, apoE-4/4 and apoE-4/3 phenotypes are related with the highest absorption efficiency of cholesterol in adults (Kesäniemi et al. 1987), in children (Tammi et al. 2001), and in FH (Gylling et al. 1989a), while those with apoE-2 phenotype have been reported to absorb less cholesterol. However, the relationship between apoE phenotype and cholesterol absorption could not be seen in some other studies where there were different intakes of dietary cholesterol (Sehayek et al. 1998, von Bergmann et al. 2003) and in subjects consuming a diet low in cholesterol (Miettinen et al. 1992a, Bosner et al. 1999).

The action of the ABC transporters is mediated by a nuclear hormone receptor called liver X receptor (LXR), which forms a heterodimer with another receptor, retinoid X receptor (Repa et al. 2000, Repa et al. 2002). These nuclear receptors are activated by oxysterols, thereby controlling the sterol absorption. The role of ABCG5 and ABCG8 in the regulation of sterol absorption has been confirmed by the study by Yu et al (2002b), which showed that the overexpression of the two transporter genes reduced the fractional absorption of dietary cholesterol. In addition, polymorphisms in gene coding for ABCG8 have been found to affect the serum plant sterol levels (Berge et al. 2002), suggesting that sterol absorption is heritably controlled.

Phytosterols interfere with cholesterol absorption (Grundy et al. 1969). The effects and mechanisms are described in detail later. However, even without added dietary phytosterols, the intake of phytosterols correlated negatively with the cholesterol absorption efficiency, thus contributing to lower total and LDL cholesterol levels (Miettinen et al. 2000b). This result indicates that phytosterols of normal food explain some variability of cholesterol absorption efficiency.

In earlier studies with hypercholesterolemic and FH subjects, lovastatin and pravastatin decreased the cholesterol absorption efficiency (Miettinen et al. 1990a, Miettinen 1991, Vanhanen et al. 1992), while in another study there was no change in cholesterol absorption after pravastatin treatment (Vanhanen and Miettinen 1995). The reduced cholesterol absorption efficiency by statins was thought to be mediated by reduced ACAT activity in enterocytes (Ishida et al. 1989) and/or by reduced synthesis of bile acids (Miettinen 1991). However, in a recent study, atorvastatin increased the intestinal cholesterol absorption in subjects with type 2 diabetes who had low baseline absorption of cholesterol (Miettinen and Gylling 2003). Thus, it remains to be determined whether the effect of statins on cholesterol absorption depends on the statin used, or is it attributable to baseline cholesterol metabolism. The statin treatment leads to increased serum plant sterol levels (Uusitupa et al. 1992, Miettinen et al. 2000a), but the effect of statins on phytosterol absorption has not been studied.

Ezetimibe is the first of a new class of the cholesterol absorption inhibitors. Recent studies suggest that Niemann-Pick C1 like 1 protein (Altmann et al. 2004) or a complex of annexin2 and caveolin proteins (Smart et al. 2004) are the possible targets

for the action of this drug. Ezetimibe inhibits cholesterol absorption by 54% compared with placebo with ~20% reduction in LDL cholesterol (Sudhop et al. 2002b). Due to the compensatory increase of cholesterol synthesis, ezetimibe is recommended to be used in combination with statins. Other cholesterol absorption inhibitors such as ACAT and MTP inhibitors have also been under recent pharmacological development (Sudhop and von Bergmann 2002).

The cholesterol absorption efficiency correlated positively with serum total and LDL cholesterol levels in a random Finnish male population (Kesäniemi and Miettinen 1987, Miettinen and Kesäniemi 1988, Miettinen and Kesäniemi 1989) but not in postmenopausal women (Karjalainen et al. 2000). In addition, there was no association between the cholesterol absorption efficiency and serum lipids in recent studies (Sehayek et al. 1998, Bosner et al. 1999), probably due to large variability in cholesterol absorption efficiency and the use of different methods. However, only insignificant correlations between cholesterol synthesis and serum lipid levels have been reported (Miettinen and Kesäniemi 1989, Miettinen et al. 1990b), suggesting that cholesterol absorption is a more important determinator of serum lipid levels than cholesterol synthesis. Thus, the factors regulating the cholesterol absorption may also have an effect on serum total and LDL cholesterol levels.

2.3.3. Measurement of sterol absorption

Sterol absorption can be measured by several different methods, mostly based on the use of radioactive compounds (Borgström 1969, Quintão et al. 1971b, Sodhi et al. 1974, Crouse and Grundy 1978, Samuel et al. 1978), and thus these are not at all suitable e.g., for children. Even though a recently developed method to study cholesterol absorption by stable isotopes (Bosner et al. 1993, Lütjohann et al. 1993) avoids the use of radioactive tracers, the measurement of cholesterol absorption by this method is still laborious. The ratios of serum plant sterols and cholestanol, a metabolite of cholesterol, to cholesterol are positively correlated with the cholesterol absorption efficiency measured by sterol balance technique (Tilvis and Miettinen 1986, Miettinen et al. 1989, Miettinen et al. 1990b) and are thus called cholesterol absorption markers. Accordingly, measurement of these non-cholesterol sterols by gas liquid chromatography (GLC) provides a simple method to assess cholesterol absorption and is especially suitable in large-scale studies and for detecting changes in cholesterol absorption during experimental studies.

2.3.4. Cholestanol

Cholestanol is a 5α-saturated derivative of cholesterol. Less than 2 mg/day cholestanol is present in the Finnish diet, and the absorption of cholestanol is only ~9% (Blomstrand and Gürtler 1968). Endogenous synthesis of cholestanol from cholesterol occurs in liver at a rate of ~12 mg/day. In serum, cholestanol is transported in lipoproteins, mainly in LDL (Salen and Grundy 1973). Elimination of cholestanol occurs via bile as such or as its acidic conversion products (Salen and Grundy 1973).

Considering the low dietary intake and low absorption of cholestanol in conjunction with the finding that during consumption of a cholestanol-free diet, the serum cholestanol levels remained fairly stable, it seems that most of serum cholestanol is derived from endogenous cholestanol synthesis. However, it has been shown that serum levels of cholestanol are heritably controlled (Gylling and Miettinen 2002b), and that polymorphisms in ABCG8 contributes to serum cholestanol levels (Berge et al. 2002). This would suggest that serum cholestanol levels are regulated by intestinal absorption and biliary secretion, since ABC transporters regulate these two metabolic events. In fact, the study by Yu et al. (2002a) revealed that in ABCG5/G8 knockout mice in addition to plant sterols, the absorption of dietary cholestanol was increased. In addition, reduction of biliary secretion of sterols by ursodeoxycholic acid increased the serum cholestanol levels (Lindenthal et al. 2002), indicating that biliary excretion of cholestanol has a role in the regulation of its serum levels. Thus, the regulation of serum cholestanol levels is not totally understood, but it seems that ABCG transporters in intestine and liver could at least partly regulate the serum levels of cholestanol. Accordingly, upregulation of intestinal ABCG5 and/or G8 would lead to decreased absorption of cholesterol as well as cholestanol. Subsequently, the serum levels of cholestanol would be decreased, and thus could be used as a marker of decreased cholesterol absorption. This would provide one explanation for the correlation between serum ratios of cholestanol and cholesterol absorption efficiency (Miettinen et al. 1989).

Serum plant sterol ratios are not reliable markers of cholesterol absorption efficiency during consumption of dietary phytosterols (Vanstone and Jones 2004). Instead, measurement of serum cholestanol ratios does offer a method to evaluate cholesterol absorption efficiency also in that state. Accordingly, cholesterol malabsorption induced by consumption of phytosterol esters leads to a ~6-15% decrease in serum cholestanol to cholesterol ratios (Gylling and Miettinen 1994b, Miettinen and Vanhanen 1994a, Gylling et al. 1997, Gylling et al. 1999, Hallikainen et al. 2000), also in FH children (Gylling et al. 1995, Vuorio et al. 2000) and FH adults receiving statin treatment (Vuorio et al. 2000). However, also non-significant changes in serum cholestanol ratios have been reported (Vanhanen and Miettinen 1992b, Vanhanen et al. 1993, Vanhanen et al. 1994).

Statin treatment, on the other hand, increases the serum ratios of cholestanol to cholesterol (Miettinen and Gylling 2003, Miettinen et al. 2003), probably by inducing the increase in cholesterol absorption (Miettinen and Gylling 2003). However, the increase in serum cholestanol ratios to cholesterol was diminished by unknown reasons in the course of a 5-year study with simvastatin (Miettinen et al. 2000a). High serum baseline cholestanol, reflecting high cholesterol absorption and low cholesterol synthesis, predicted a poor response to statin treatment with increased risk for recurrent coronary events in a long-term study with simvastatin compared with the subjects with low baseline cholestanol levels (Miettinen et al. 1998). This suggests that a subgroup of patients not responding to statin treatment could be revealed by measuring serum cholestanol levels. This would offer an opportunity to treat such patients with a combination of statins and cholesterol absorption inhibitors.

2.4. Postprandial lipoproteins

Postprandial lipoproteins are reflections of the lipids appearing in blood circulation mostly in CM and CM remnants after a fatty meal. Many genetic, environmetal, and dietary factors regulate the elimination of postprandial lipids. Since 1970's it has been known that postprandial lipids are associated with CHD (Zilversmit 1979). Further, it was shown that especially the remnants of TRL were atherogenic (Havel 1994, Karpe 1999). VLDL synthesized by the liver with apoB-100 as its structural protein has the same density as CM remnants with apoB-48. Lipoproteins with apoB-48 and apoB-100 compete for the same lipolytic pathway (Karpe et al. 1993, Björkegren et al. 1996) which explains the significant increase in apoB-100 containing lipoproteins in postprandium (Cohn et al. 1988, Karpe et al. 1993). Thus, in addition to apoB-48 containing lipoproteins, TRL in postprandial serum include also apoB-100 containing lipoproteins.

2.4.1. Liver receptors

After formation of CM by the enterocyte, they are released and lipolyzed by LPL, resulting in the formation of CM remnants. The clearance of CM remnants is dependent on apoE, which is bound to heparan sulfate proteoglycans (HSBG) in the space of Disse (Cooper 1997). In addition to apoE, HL facilitates the endocytosis of remnants to different receptors involved in uptake of CM remnants. The most important receptors are LDL apoB receptor and LDL receptor-related protein (LRP), but also other receptors, such as liver cell remnant receptor, asialoglycoprotein, and lipolysis stimulated receptor have been reported to participate in this process (Cooper 1997, Mahley and Ji 1999). In addition, remnants may be taken up directly by HSBG. In mice, ~50-60% of the CM remnant clearance is thought to be mediated by LDL apoB receptor (Cooper 1997), but the role of different receptors in the clearance of remnants in humans is unclear.

2.4.2. Measurement of postprandial lipoproteins

Vitamin A added to fat meals can label postprandial lipoproteins (Hazzard and Bierman 1976, Weintraub et al. 1987), but it was found also in more dense lipoproteins. Thus, apoB-48 was suggested to be a more reliable marker of postprandial lipoproteins (Krasinski et al. 1990, Karpe et al. 1993). Due to laborious determination of apoB-48, simple markers for postprandial lipoproteins were needed. Accordingly, in addition to single measurement of apoB-48 (Smith et al. 1999) and remnant-like particle (RLP) cholesterol (Nakajima et al. 1993), squalene was found to reflect postprandial lipoproteins (Gylling and Miettinen 1994a, Rajaratnam et al. 1999). In fact, squalene labeled postprandial lipoproteins even more specifically than vitamin A, and the postprandial curves resembled those of apoB-48 (Rajaratnam et al. 1999). Most recently, a breath test using a stable isotope was validated to provide information about CM remnant metabolism (Redgrave et al. 2001).

CM-like emulsions have been used to assess more accurately the metabolism of TRL (Hallberg 1965, Carlson and Rössner 1972), and in this way one can avoid the intra-individual differences in intestinal absorption of lipids. One example of these

emulsions is commercially available Intralipid, which is rich in plant sterols (Carlson and Rössner 1972, Björkegren et al. 1996). In plasma, apolipoproteins from circulating lipoproteins become attached to the emulsions (Carlson 1980), and this particle activates LPL. The metabolism of the CM-like emulsion resembles mostly the metabolism of CM (Carlson and Rössner 1972, Redgrave et al. 1987), even though also slower clearance rates for emulsions have been reported (Hultin et al. 1995). Double-labeling of the CM-like emulsions revealed diminished removal of postprandial lipoproteins in CHD (Maranhão et al. 1996) and their enhanced removal during lipid-lowering medication (Santos et al. 2000). To avoid the use of radioactive labels, a second possibility is to add squalene to Intralipid in order to measure postprandial lipoproteins (Relas et al. 2001b).

2.5. Phytosterols in blood

2.5.1. Serum and lipoprotein phytosterols

Due to their low absorption and effective excretion, only small amounts (~0.7-1.0 mg/dl) of phytosterols may be detected in normal human serum, thus accounting for less than 1% of respective cholesterol levels (Salen et al. 1970, Tilvis and Miettinen 1986, Björkhem et al. 2001). In order to eliminate the influence of varying cholesterol concentrations, the phytosterol values are usually standardized and expressed, in addition to concentrations, also as ratios to cholesterol, in terms of 10^2 x mmol/mol of cholesterol. In a random Finnish adult population aged 52 years, the average serum sitosterol ratio was 122±11 10^2 x mmol/mol of cholesterol and serum campesterol ratio 190±17 10^2 x mmol/mol of cholesterol (Nissinen et al. 2000). An extensive inter-individual variation exists in serum plant sterol levels, which were recently found to be heritably controlled (Gylling and Miettinen 2002b) by polymorphisms in the ABCG8 transporter gene (Berge et al. 2002). Hypercholesterolemic subjects, such as subjects with FH, have elevated concentrations of serum plant sterols, probably due to increased amounts of LDL. However, even when adjusted to the respective cholesterol values, the levels of plant sterols in serum were higher in the FH men compared with the randomly selected healthy men (Gylling and Miettinen 1988). In serum, LCAT is capable of esterifying plant sterols (Nordby and Norum 1975) and the esterification percentage of plant sterols resembles that of cholesterol. Accordingly, approximately 70% of plant sterols in serum are transported in an esterified form (Salen et al. 1970, Tilvis and Miettinen 1986). Plant stanols are present in serum only in trace amounts (~0.03 mg/dl), and they are usually detectable only after consumption of plant stanol esters (Gylling et al. 1999).

Only a few studies have defined the distribution of phytosterols in different lipoproteins in humans. The concentrations of plant sterols seem to parallel those of cholesterol. Thus, LDL and HDL are the major transporters of phytosterols. However, as ratios to cholesterol, plant sterols are mostly accumulated in HDL in subjects with familial hypertriglyceridemia (Tilvis and Miettinen 1986), in FH with and without ileal bypass (Koivisto and Miettinen 1988a), and in type 2 diabetes (Miettinen and Gylling 2003). On the other hand, the lowest ratios of plant sterols were seen in VLDL. It has been suggested that the high ratios of plant sterols in HDL could be

associated with reverse cholesterol transport by HDL, as shown in sitosterolemia (Robins and Fasulo 1997).

2.5.2. Red cell phytosterols

The red cell has a complex cell membrane composed of mainly lipids and proteins and an interior containing metabolic mechanisms e.g., those involved in hemoglobin synthesis (Mohandas and Morrow 2000). The lipid compartment of the membrane contains in addition to phospholipids and free cholesterol also other sterols, including plant sterols (Kuksis et al. 1976). Since red cells are not able to synthesize sterols, all the sterols in the cell membrane must be obtained from the plasma. Indeed, sterols are freely exchanged and there seems to be an equilibrium between plasma and red cells (Ashworth and Green 1964, Salen et al. 1970). Cholesterol is taken up by the red cells more readily than the plant sterols (Edwards and Green 1972, Child and Kuksis 1982, Child and Kuksis 1983), possibly due to the longer side chain present in plant sterols. However, other sterols, including plant sterols and lathosterol, may replace a small amount (~20-30%) of cholesterol in red cells (Bruckdorfer et al. 1969, Edwards and Green 1972). The regulated assembly of proteins and lipids in the membrane leads to the unique shape of the red cell and its ability to become deformed during its passage through the narrow blood vessels in the circulation. Any disruption to the structure of the red cell, as a result of changes in any of its components, may lead to altered cell shape, deformability characteristics, and overall hemorrheology properties. Thus, increased levels of cholesterol in red cells in hypercholesterolemic subjects may lead to increased osmotic fragility and decreased deformability (Kanakaraj and Singh 1989, Kohno et al. 1997). Statins decrease the cholesterol level in red cells and result in improvement of reduced red cell deformability and blood rheology (Martínez et al. 1996, Kohno et al. 1997, Koter et al. 2002).

In phytosterolemia the total plant sterol content in red cells is high (12-13 mg/dl) (Bhattacharyya and Connor 1974), possibly causing the episodes of hemolysis (Björkhem et al. 2001) due to the increased fragility of the red cells (Bruckdorfer et al. 1969). In addition, in stroke-prone spontaneously hypertensive rats, plant sterol-enriched diet increased the red cell concentrations of plant sterols and made the red cells less deformable (Ratnayake et al. 2000, Naito et al. 2003). In rats the increase of plant sterols in red cells by feeding a sterol-enriched diet was three to four times higher than the respective increase of plant stanols from a stanol-enriched diet (Ratnayake et al. 2003). In addition, the deformability of the red cells was lower by the sterol-enriched diet than with the stanol-enriched diet. Hence, the increase of plant sterols in red cells seems to contribute to their reduced deformability. However, the life span of the rats was reduced even more with the plant stanol- than with the plant sterol-enriched diet (Ratnayake et al. 2003). Moreover, in apoE-deficient mice, a reduction of serum cholesterol by plant sterols appeared to be protective to their red cells (Moghadasian et al. 1999b). There is little information about the effects of consumption of phytosterols on the red cell phytosterol levels in humans. In a recent study, consumption of 1.6 g/day of plant sterols esters by 89 adult hypercholesterolemic subjects increased the total plant sterol concentrations in red cells by ~80% without having any effect on red cell deformability (Hendriks et al.

2003). The dietary plant sterol esters did not affect red cell cholesterol concentrations, and thus it was concluded that plant sterols could not replace cholesterol in red cell membranes.

2.5.3. Effects of dietary phytosterols on their serum levels

Dietary intake of plant sterols reflects their serum levels (Salen et al. 1970, Vanhanen and Miettinen 1992b). In vegetarians who have a high dietary intake of plant sterols, serum levels of plant sterols can be doubled compared with controls (Vuoristo and Miettinen 1994). The effect of dietary phytosterols on the respective levels in serum has been evaluated in several studies, but no data exist on the phytosterol distribution in different lipoproteins during phytosterol-enriched diet.

Plant sterols, even from normal food, affect their serum levels, since consumption of rapeseed oil spreads, containing naturally ~360 mg/100 g of plant sterols, increases the levels of serum plant sterols (Vanhanen and Miettinen 1992b, Vanhanen et al. 1993, Miettinen and Vanhanen 1994b). The major increase (up to 50-60%) occurs in campesterol compared with an up to 20% increase in sitosterol.

Phytosterol-enriched diet

The effect of phytosterol-enriched diet on serum levels of phytosterols has been studied by adding free or esterified phytosterols to the diet with daily doses ranging from 0.7 g up to 18 g. In earlier studies with free plant sterols, daily intake of 18 g of soy derived plant sterols led to extremely high serum plant sterol levels (Lees et al. 1977). Accordingly, the serum campesterol concentration increased up to 21 mg/dl and sitosterol up to 0.8 mg/dl. However, in the same study, daily consumption of 3g of tall oil sterols led to only a moderate increase (up to 2.5 mg/dl) in plasma plant sterol levels. Even smaller doses (0.7-1 g/day) of dietary sitosterol resulted in a 40% increase in plasma sitosterol levels (Vanhanen and Miettinen 1992b, Miettinen and Vanhanen 1994a). In infants fed commercial formulas enriched with vegetable oils containing 300-400 mg/day of plant sterols, the serum total plant sterol concentration was increased up to 9 mg/dl (Mellies et al. 1976a). The authors speculated that children have a limited capability to discriminate between these sterols in terms of their absorption. On the other hand, consumption of 12 g/day of free sitosterol increased the serum sitosterol levels only up to 1.5 mg/dl in children and adolescents aged 8 to 20 years (Schlierf et al. 1978), and only a 14-22% increase in serum sitosterol was reported in children aged 5 to 11 and 10 to 14 years during daily consumption of 6 g of free sitosterol (Becker et al. 1992, Becker et al. 1993). However, there are no studies evaluating the effects of the dietary plant sterols in their esterified form on serum plant sterol levels in children.

In adults, consumption of plant sterol esters with doses of 1.6-2.4 g/day increased the levels of serum campesterol by 50-90% and serum sitosterol by 35-40% in different hypercholesterolemic populations (Weststrate and Meijer 1998, Jones et al. 2000, Hallikainen et al. 2000, Nestel et al. 2001, Mussner et al. 2002, Hendriks et al. 2003). The absolute increase was highest in subjects with the highest baseline serum plant sterol levels (Hendriks et al. 2003). Despite the marked percentual increase of serum

plant sterol levels during consumption of plant sterol ester products, the mean concentrations of total plant sterols have remained $\leq$ 2.0 mg/dl, even in subjects with heterozygous phytosterolemia (Stalenhoef et al. 2001, Kwiterovich et al. 2003).

On the other hand, since plant stanols inhibit also the absorption of plant sterols, the consumption of free (Vanhanen and Miettinen 1992b, Becker et al. 1993, Miettinen and Vanhanen 1994a) and esterified (Vanhanen et al. 1993, Vanhanen et al. 1994, Gylling et al. 1995, Gylling et al. 1999, Hallikainen et al. 2000) plant stanols decrease the serum levels of both campesterol and sitosterol by 15-45%. The decrease of serum plant sterols is negatively correlated with the respective baseline levels, suggesting that serum plant sterols are decreased more in subjects with high baseline plant sterol levels and high sterol absorption (Vanhanen et al. 1993, Gylling et al. 1995, Gylling et al. 1997, Vuorio et al. 2000). The percentual increase of serum plant stanols by consumption of plant stanols is even higher than that of plant sterols during consumption of a sterol-enriched diet. However, the actual amounts remain very small, ranging from 11 μg/dl to 16 μg/dl for campestanol and from 27 μg/dl to 53 μg/dl for sitostanol (Gylling et al. 1999, Hallikainen et al. 2000).

2.5.4. Phytosterol elimination

The absorbed phytosterols are mostly eliminated through bile, even though some excretion via the skin surface may also occur (Bhattacharyya et al. 1983). The secretion of campesterol and sitosterol through bile occurs along with cholesterol and is dependent on ABCG5 and ABCG8 (Berge et al. 2000). Biliary secretion of plant sterols is more rapid than that of cholesterol (Salen et al. 1970, Sudhop et al. 2002c), and campesterol is excreted slower than sitosterol (Sudhop et al. 2002c). In addition, the turnover of plant stanols is more rapid than that of plant sterols (Ostlund et al. 2002). Thus, in addition to low absorption, the rapid excretion of phytosterols contributes to their low serum concentrations. Conflicting results exist on whether plant sterols can be converted into acidic products. In an earlier human study (Salen et al. 1970), sitosterol was converted to bile acids, but this has not been confirmed in further studies (Boberg et al. 1990). The unabsorbed plant sterols pass through the intestine and are partly converted to bacterial products, coprostanols and coprostanones (Miettinen et al. 1965, McNamara et al. 1981), whereas the unabsorbed plant stanols seem to be quite resistant to bacterial action.

2.5.5. Effects of lipid-lowering medications on serum phytosterols

2.5.5.1. Statins

After an initial decrease due to the marked decrease in LDL cholesterol levels, the concentrations of plant sterols are increased during statin treatment (Miettinen et al. 2000a). In addition, statins cause a consistent increase in the ratios of serum plant sterols to cholesterol in short- (Uusitupa et al. 1992, Vanhanen et al. 1992, Vanhanen and Miettinen 1992a) and long-term studies (Miettinen et al. 2000a, Miettinen and Gylling 2003, Miettinen et al. 2003). Long duration (Miettinen et al. 2000a) and high dose (Miettinen et al. 2003) statin treatment, and higher baseline absorption of sterols (Miettinen et al. 2000a) are the major factors predicting the increase of plant sterols

during statin treatment. The increased serum plant sterol levels by statins are most likely due to diminished excretion of plant sterols through bile (Miettinen 1991). Additionally, reduced biliary excretion of cholesterol (Gylling and Miettinen 1996) depletes the intestinal cholesterol pool, most likely favoring the absorption of dietary plant sterols (Miettinen and Gylling 2003). In fact, decreased biliary excretion of sterols has been shown to be related to the increased serum levels of plant sterols (Miettinen et al. 1990b).

Two studies conducted with hypercholesterolemic subjects, including those with FH, have evaluated the effects of combination of statins and dietary plant sterol esters on serum plant sterol levels (Neil et al. 2001, O'Neill 2003). Accordingly, daily doses of 1.4 g and 2.5 g of dietary plant sterols revealed ~40% and ~20% increase in serum campesterol and sitosterol levels, respectively, when compared with baseline values (O'Neill 2003) or with the placebo group (Neil et al. 2001). On the other hand, by combining plant stanol esters to statin treatment, the serum levels of both campesterol and sitosterol can be decreased by about 30% (Vanhanen 1994, Gylling and Miettinen 1996, Vuorio et al. 2000, Gylling and Miettinen 2002a, Gylling and Miettinen 2002c). Combination of a statin and plant stanols results in even lower levels of serum plant sterols than were present at baseline before the statin treatment was started. Accordingly, plant stanol esters prevent the increase of serum plant sterol levels caused by statin treatment.

2.5.5.2. Other lipid-lowering medications

Bile acid sequestrants increase the fecal elimination of cholesterol as bile acids and decrease the absorption of cholesterol (McNamara et al. 1980, Gylling et al. 1989b). However, with respect to the absorption markers, serum plant sterol levels are increased in monotherapy (Gylling et al. 1989b) as well as in combination with statins (Uusitupa et al. 1992). This increase is thought to be caused by reduced biliary excretion of plant sterols. However, in phytosterolemic subjects, the serum plant sterol levels are efficiently decreased by bile acid sequestrants due to enhanced fecal elimination of plant sterols and sequentally increased synthesis of bile acids (Björkhem et al. 2001).

Fibrates decrease the serum ratios of plant sterols to cholesterol by about 30-40% due to enhanced biliary excretion and subsequently decreased absorption of sterols (Miettinen et al. 1992b, Vanhanen and Miettinen 1995). However, in combination with statins, the serum levels of plant sterols return to levels obtained at baseline (Miettinen et al. 1992b, Vanhanen and Miettinen 1995). The newest lipid-lowering drug, ezetimibe, inhibits the absorption of cholesterol, and recently was shown to decrease serum campesterol and sitosterol levels by 48% and 41%, respectively, in hypercholesterolemic subjects (Sudhop et al. 2002b).

2.5.6. Phytosterolemia

Phytosterolemia, or sitosterolemia, is a rare (prevalence 1:1 000 000) recessively inherited disease characterized by high serum plant sterols levels accompanied by subcutaneous and tendon xanthomas, episodes of hemolysis and arthralgias, and most

importantly development of atherosclerosis at a young age (Bhattacharyya and Connor 1974, Björkhem et al. 2001). The genetic basis of this disease was recently resolved by identifying mutations in genes coding ABC transporters G5 and G8 (Berge et al. 2000, Lu et al. 2001) located in chromosome 2p21 (Patel et al. 1998). These two genes are expressed in the intestine and liver (Berge et al. 2000), thus contributing to the fundamental metabolic defects in phytosterolemia; increased sterol absorption and decreased sterol excretion through bile (Miettinen 1980, Björkhem et al. 2001). Accordingly, serum contains extremely high concentrations of serum plant sterols, ranging from 20 mg/dl to 94 mg/dl (Björkhem et al. 2001, Berge 2003). The most abundant plant sterol in serum is sitosterol, whereas campesterol concentrations amount to approximately half of those of sitosterol (Salen et al. 1985, Lütjohann et al. 1995). Most patients have also increased serum levels of plant stanols and cholestanol (Salen et al. 1985, Björkhem et al. 2001). The phytosterols are mainly (75-85%) transported in LDL (Bhattacharyya and Connor 1974, Miettinen 1980, Salen et al. 1985), while HDL contains mostly the remainder. In addition to increased serum levels, plant sterols are also accumulated in red cells (Bhattacharyya and Connor 1974), which is the probable reason for hemolysis and increased fragility of the red cells (Björkhem et al. 2001).

In homozygous patients, the absorption of sitosterol is increased by up to ~60% while the absorption of cholesterol is reported to be only slightly higher than in controls and mostly within the high normal range (Björkhem et al. 2001). This slight increase in cholesterol absorption and reduced biliary output leads to moderately increased serum cholesterol levels in most subjects. Heterozygous subjects are clinically normal, in comparison with control subjects, they have only slightly higher serum plant sterol levels due to the slight increase in plant sterol absorption (Salen et al. 1992). The higher increase of serum plant sterols by heterozygotes is avoided by the extensive excretion of plant sterols into bile (Salen et al. 1992).

A diet low in cholesterol and plant sterols (Miettinen 1980, Nguyen et al. 1991, Cobb et al. 1997) usually results in a rapid decrease in serum plant sterol levels. In addition, cholestyramine (Miettinen 1980, Salen et al. 1985, Nguyen et al. 1991), and plant stanols (Lütjohann et al. 1995) have been used as strategies to decrease serum plant sterols. The recent study by Salen et al. (2004) showed that ezetimibe decreased serum sitosterol and campesterol levels by 21% and 24%, respectively, in phytosterolemic patients, providing a new tool for treatment of these patients.

2.5.7. Phytosterols and CHD

The finding of phytosterolemia led to speculations that plant sterols may have a role in atherogenesis. Plant sterols are found in human atheromas even in non-phytosterolemic subjects, even though their concentrations are lower than that of cholesterol (Mellies et al. 1976b). However, plant sterol feeding in different animal studies (hamsters, mice, and rabbits) has revealed prevention and regression of atherosclerotic lesions (Ikeda et al. 1981, Moghadasian et al. 1997, Moghadasian et al. 1999a, Ntanios et al. 2003). Similar results were obtained by feeding plant stanols to mice and rabbits (Ntanios et al. 1998, Volger et al. 2001). In one study in rabbits, sitostanol was more effective than sitosterol at preventing the formation of atheromas

(Ikeda et al. 1981). Furthermore, it was recently shown that only trace amounts of plant sterols and stanols were deposited in the aortas of rabbits fed sitosterol and sitostanol preparations (Kritchevsky et al. 2003).

In humans, Glueck and co-workers (1991) demonstrated that high serum plant sterol levels were associated with increased premature CHD independent of serum cholesterol levels. In addition, in subjects admitted to elective coronary artery bypass graft operation, a high serum plant sterol level predicted a positive family history of CHD (Sudhop et al. 2002a). Further, it was shown that a high plasma sitosterol level was associated with the increased extent and severity of angiographically measured CHD (Sutherland et al. 1998), and that increased ratios of serum plant sterols to cholesterol were independent risk factors of CHD in a randomly selected population of postmenopausal women (Rajaratnam et al. 2000). These findings were recently confirmed in a long-term follow-up population study where an increased risk of coronary events was noted in the fourth quartile of serum sitosterol with the mean concentration of >0.2 mg/dl as compared with other quartiles (Assmann et al. 2003). Thus, there is accumulating indirect evidence from human studies to suggest that there is an association between serum plant sterol levels and CHD. Whether this association is solely due to increased cholesterol absorption (indicated by high serum plant sterol ratios) or whether plant sterols have an independent role in development of atherosclerosis is not known.

2.6. Cholesterol lowering effects of dietary phytosterols

2.6.1. A short history

The hypocholesterolemic property of dietary plant sterols was demonstrated over 50 years ago (Pollak 1953). Earlier human studies consisted of interventions using mostly large daily doses (3-18 g) of plant sterols in their unesterifed form resulting in a mean of 10-20% reduction in serum cholesterol concentrations (Lees et al. 1977, Pollak and Kritchevsky 1981, Pollak 1985) due to inhibition of cholesterol absorption (Grundy et al. 1969). The poor solubility and the increase in serum plant sterol levels due to the high intake of free plant sterols led to preparations of plant sterol esters (Mattson et al. 1977, Mattson et al. 1982). These esterified sterols lowered serum cholesterol levels similarly as the free sterols even though there was a slightly lower inhibition of cholesterol absorption (33% reduction by esterified vs. 42% reduction by free sterols).

Plant stanols in an unesterified form were synthesized from plant sterols, and in experimental animals were shown to decrease serum cholesterol levels even more efficiently than plant sterols (Sugano et al. 1977, Ikeda et al. 1981). However, in the study by Denke (1995), plant stanols in a poorly fat-soluble form even at a high dose (3 g/day) did not evoke any reduction in serum cholesterol. Thus, to make plant stanols more fat-soluble, they were transesterified making them more easily dissolvable in dietary oil and spreads. Accordingly, subsequent several short-term studies (Vanhanen et al. 1993, Miettinen and Vanhanen 1994a, Gylling and Miettinen 1994b, Vanhanen et al. 1994) and one long-term (Miettinen et al. 1995) study using plant stanol esters with varying doses (0.8-3.4 g/day) led to 5-15% decrease in LDL

cholesterol. These studies were followed by the commercial marketing of a plant stanol ester spread in 1995 followed by the marketing of a plant sterol ester spread in the late 1990's. Short-term studies conducted with both plant stanol and sterol ester spreads reported similar hypolipidemic effects with both spreads in adults (Weststrate and Meijer 1998, Hendriks et al. 1999, Hallikainen et al. 2000).

2.6.2. Hypolipidemic mechanisms of phytosterols

Phytosterols in their free (Grundy et al. 1969, Lees et al. 1977, Sugano et al. 1977, Ikeda et al. 1988b, Vanstone et al. 2002) and esterified forms (Mattson et al. 1977, Mattson et al. 1982, Vanhanen et al. 1994, Jones et al. 2000) inhibit cholesterol absorption. The earlier studies hinted at more effective inhibition of cholesterol absorption by plant stanols compared with plant sterols (Sugano et al. 1977, Heinemann et al. 1991), but recent studies with plant stanol and sterol esters have reported similar, ~30%, inhibition of cholesterol absorption (Jones et al. 2000, Normén et al. 2000). The precise mechanism behind the lowered cholesterol absorption is not fully understood, but the reduced micellar solubilization of intestinal cholesterol is thought to be the major mechanism behind the inhibition of cholesterol absorption (Slota et al. 1983, Armstrong and Carey 1987, Ikeda et al. 1988b, Heinemann et al. 1991, Nissinen et al. 2002). Earlier it was proposed that phytosterols would interfere with the esterification of cholesterol in the enterocyte, but this hypothesis was not confirmed, e.g., sitosterol did not compete with cholesterol for cholesterol esterase or ACAT-catalyzed esterification (Field and Mathur 1983, Field et al. 1997). Recently, it was reported that plant stanols administered once daily were as effective as three separate doses (Plat et al. 2000). The authors suggested that in addition to inhibition of the micellar solubility of cholesterol, plant stanols could have another mechanism for inhibition of cholesterol absorption. In fact, consumption of plant stanols increased the expression of intestinal ABCA1 (Plat and Mensink 2002b). The authors speculated that since ABCA1 has been shown to have a role in sterol absorption, probably by mediating the efflux of sterols from the enterocyte, an upregulation of the transporter induced by plant stanols would be another mechanism for inhibition of cholesterol absorption.

The inhibition of cholesterol absorption by phytosterols leads to diminished amounts of cholesterol in the circulation and liver. The plant stanol-induced decrease in liver cholesterol led to increased LDL apoB receptor mRNA concentrations (Plat and Mensink 2002a), allowing increased uptake of cholesterol into the liver. In fact, the conversion of VLDL and IDL to LDL was reduced in type 2 diabetes during consumption of a plant stanol ester-enriched diet, suggesting that the fractional catabolic rate of VLDL and IDL was effective (Gylling and Miettinen 1996).

Subcutaneously or intravenously injected phytosterols lower serum cholesterol concentrations in experimental animals (Konlande and Fisher 1969, Vanstone et al. 2001). This has led to speculations that other mechanisms, in addition to inhibition of cholesterol absorption, could contribute to reduced serum cholesterol levels. The proposed mechanisms include modification of enzymes acting on cholesterol metabolism, such as hepatic acetyl CoA carboxylase (Laraki et al. 1993). However, most probably injected phytosterols, even if only present in small quantities, are

transported via bile into the intestinal lumen where they interfere with cholesterol absorption.

2.6.3. Dietary phytosterols and serum lipids

Adults

The results from 41 randomized trials conducted in subjects in an age range of 4 to 60 years describing the effects of dietary phytosterols as cholesterol lowering agents were summarized recently by Katan and colleagues (2003). The lowest dose of phytosterols used, mostly in esterified form, was 0.7 g/day, but most studies used amounts of 1.5 to 3.0 g/day added most frequently to spread, but also to mayonnaise, butter, olive oil, and to some lower-fat foods, such as ground beef. The placebo-adjusted reduction of LDL cholesterol was 10% with both plant stanols and sterols, and no differences were reported between the sterol and stanol products. In fact, closer inspection of the results from human comparative studies conducted with either free (Miettinen and Vanhanen 1994a, Vanstone et al. 2002) or with esterified plant stanols and sterols (Weststrate and Meijer 1998, Jones et al. 2000, Hallikainen et al. 2000, Noakes et al. 2002) revealed similar mean reductions of around 11% in LDL cholesterol. The absolute, but not percentual, reduction in LDL cholesterol increases with age (Katan et al. 2003). This could simply be due to the fact that the decrease in LDL cholesterol is higher in those subjects with higher baseline LDL cholesterol levels (Miettinen et al. 1995). The percentage reduction in LDL cholesterol is dose dependent, but only slight further reductions were reported with doses higher than 2.5 g/day (Katan et al. 2003). According to original data from several studies reviewed by Katan et al. (2003), serum HDL cholesterol and TG remain mainly unchanged during consumption of phytosterols. In addition, no (Hallikainen et al. 2000) or a slight but significant decrease (Gylling and Miettinen 1994b) in VLDL cholesterol has been reported.

Recent studies using non-fat or low fat products enriched with plant sterols have reported contradictory results. Accordingly, a 12% decrease in LDL cholesterol was obtained during 8 weeks' consumption of plant sterol-enriched (2 g/day of plant sterols) orange juice (Devaraj et al. 2004), but in another study, consumption of non-fat or low fat beverages enriched with plant sterols (1.8 g/day) for 3 weeks did not evoke any changes in serum total or LDL cholesterol when compared with placebo (Jones et al. 2003). Milk, youghurt, bread, and cereal products enriched with plant sterols all resulted in a significant reduction (5-16%) in LDL cholesterol, but it was concluded that cholesterol-lowering effect of plant sterol esters may depend on the food matrix to which they are added (Clifton et al. 2004).

Long-term studies

Only two long-term studies lasting for 1 year have been published, one with stanol esters (Miettinen et al. 1995) and the other one with sterol esters (Hendriks et al. 2003). The former study reported ~9% and ~13% reduction in LDL cholesterol with the daily doses of 1.8 g and 2.6 g of plant stanols, respectively, while the latter resulted in respective ~6% reduction with the daily dose of 1.6 g of plant sterols

compared with placebo. However, closer inspection of the results revealed that in women, serum total and LDL cholesterol levels were not significantly decreased by the sterol esters, as compared with both baseline and control subjects, only in men was the LDL cholesterol significantly reduced (Brink and Hendricks 2000). Furthermore, there was a rebound effect in total and LDL cholesterol after termination of supplementing with plant sterol esters (2-month study) but the beneficial effect of plant stanol esters persisted (O'Neill 2003). In addition, a recent study in subjects with type 2 diabetes illustrated the difficulties to maintain the cholesterol lowering effect during consumption of plant sterol ester spread for 12 weeks (Lee et al. 2003). This transient effect has been suggested to be attributable to diminished bile acid synthesis (O'Neill 2003) and/or increased cholesterol synthesis, as shown by increased serum levels of cholesterol precursor sterols.

Adults with FH

Only one study has reported the effects of phytosterols in FH as monotherapy. In that small group of FH adults (n=4), consumption of stanol ester spread (2.2 g of stanols/day) for 3 months resulted in 11% lowering in LDL cholesterol levels (Vuorio et al. 2000).

Children

In healthy children, consumption of plant stanol ester spread decreased LDL cholesterol by 7.5% (Tammi et al. 2000) and 15.5% (Williams et al. 1999) with daily doses of 1.5 g and 3 g of plant stanols. Earlier studies conducted with FH children who consumed free sitosterol or sitostanol for three to seven months detected a 17-30% reduction in LDL cholesterol (Becker et al. 1992, Becker et al. 1993). Sitostanol appeared to be more effective than sitosterol in the only comparative study conducted with phytosterol products in FH children (Becker et al. 1993). However, recent studies with FH children using either plant stanol (2.2-3.0 g/day) (Gylling et al. 1995, Vuorio et al. 2000) or sterol (1.6-2.3 g/day) (Amundsen et al. 2002, de Jongh et al. 2003) ester spreads for 8 to 12 weeks have reported similar reductions in LDL cholesterol, ranging from 10% to 18% compared with placebo or with baseline.

2.6.4. Cholesterol metabolism and lipid lowering properties of phytosterols

Plant stanol esters are most effective in adult subjects with high baseline absorption and low synthesis of cholesterol. In contrast, high serum levels of cholesterol precursors, reflecting high cholesterol synthesis, predicted a lesser reduction of serum cholesterol (Gylling et al. 1997, Gylling et al. 1999, Gylling and Miettinen 2002a). However, opposite findings were observed in FH children, since LDL cholesterol was decreased most in those subjects with the highest serum lathosterol and Δ^8-cholestenol levels, suggesting high efficacy of cholesterol synthesis (Gylling et al. 1995). The reason for this discrepancy is not known, i.e., more studies are required to evaluate the effect of baseline cholesterol metabolism on the response to phytosterol treatment.

Subjects with the apoE-4 phenotype showed more consistent decrease in serum cholesterol levels in some studies using plant stanol esters compared with the subjects with apoE-3/3 phenotype (Vanhanen et al. 1993, Miettinen and Vanhanen 1994a, Hallikainen et al. 2000). According to these results, it was suggested that inhibition of cholesterol absorption is most efficient in subjects with high cholesterol absorption efficiency, such as in subjects with apoE-4 phenotype (Kesäniemi et al. 1987). However, this association has not been confirmed in recent studies (Plat and Mensink 2000, Tammi et al. 2002), or during consumption of plant sterol esters (Hallikainen et al. 2000, Geelen et al. 2002).

2.6.5. Combination of statins and phytosterols

Monotherapy with statins may not be sufficiently effective in lowering LDL cholesterol in some patient groups such as in subjects with FH. In addition, the dual mechanism of combination therapy acting on inhibition of cholesterol synthesis and cholesterol absorption may provide an even more profound cholesterol lowering efficacy. Further, the analysis of one large statin trial revealed a subgroup of coronary patients, in whom recurrent coronary events were not reduced by statin treatment alone (Miettinen et al. 1998). It was shown that in those subjects, the baseline serum absorption sterol markers were higher than in the subjects responsive to statin treatment. In addition, in subjects with a higher baseline sterol absorption, statin treatment caused a larger increase of serum plant sterols compared with the subjects with low baseline sterol absorption (Miettinen et al. 2000a). Accordingly, it was suggested that these subjects would have benefited from inhibition of cholesterol absorption. In fact, it was later shown that plant stanol ester spread combined with chronic statin treatment caused a further significant decrease in serum cholesterol only in subjects with high baseline absorption (Gylling and Miettinen 2002a). The decrease of serum plant sterols was also more marked in the same patient group.

The effects of spreads enriched with phytosterols as an additional treatment with different statins in various hypercholesterolemic populations have been evaluated in the studies listed in Table 1. In all studies the previous statin treatment had been started at least for several months this being followed by addition of plant stanol or sterol ester spread to the diet. In addition, in one study cerivastatin and plant sterol ester spread were started simultaneously, and this combination led to –39% reduction in LDL cholesterol compared with –32% reduction with combination of cerivastatin and placebo (Simons 2002). The calculated mean 10-15% decrease in LDL cholesterol from the studies presented in Table 1 suggests that adding phytosterol esters to statin treatment is more effective than doubling the statin dose, which usually causes an additional lowering of LDL cholesterol levels of 5% to 7% (Jones et al. 1998). In the only comparable study using plant stanol and sterol esters (O'Neill 2003), the results from FH subjects with statin treatment and unaffected subjects without any hypolipidemic treatment were pooled, since no differences existed between the groups. In this pooled population, the effect of plant sterol ester spread disappeared during the 2-month study, leading to only a nonsignificant 3% reduction in LDL cholesterol at 8 weeks as shown in Table 1.

Table 1. Studies evaluating the lipid lowering effect of dietary plant stanol and sterol ester spreads combined with statin treatment

Age, Years[1]	*Patients n*	*Statin, dose*	*Plant stanol/sterol dose, duration*	*ΔLDL% Reference*
55	*hyperchol. n=7*	*pravastatin 40 mg/day*	*stanol ester 1.5 g/day, 6 weeks*	*-4% vs. placebo, ns. (Vanhanen 1994)*
60±2	*DM2*[2] *n=8*	*pravastatin 40 mg/day*	*stanol ester 3 g/day, 7 weeks*	*-14% vs. placebo, p<0.05 (Gylling and Miettinen 1996)*
48-56	*CHD women n=10*	*simvastatin 10-20 mg/day*	*stanol ester 3 g/day, 7 weeks*	*-16% vs. baseline, p<0.05 (Gylling et al. 1997)*
33-80	*hyperchol. n=167*	*lova-, prava-, simva-,atorva-statin n.a.*	*stanol ester 3 g/day, 8 weeks*	*-10% vs. placebo, p<0.0001 (Blair et al. 2000)*
18-41	*FH n=12*	*simvastatin 20-40 mg/day*	*stanol ester 2.2 g/day, 6 weeks*	*-20% vs. baseline, p<0.001 (Vuorio et al. 2000)*
18-69	*FH n=30*	*n.a.*	*sterol ester 2.5 g/day, 4 weeks*	*-11% vs. placebo, p<0.001 (Neil et al. 2001)*
53±15	*FH* n=65*	*n.a.*	*stanol ester 1.6 g/day stanol ester 2.6 g/day sterol ester 1.6 g/day all 8 weeks*	*-6% vs. placebo, p<0.001 -7% vs. placebo, p=0.01 -3% vs. placebo, ns. (O'Neill 2003)*
68±1	*CHD hyperchol. n=11*	*simvastatin 20 mg/day*	*stanol ester 2.25 g/day, 8 weeks*	*-13% vs. baseline, p<0.05 (Gylling and Miettinen 2002c)*

*[1] Mean±SEM or range; [2] DM2, diabetes mellitus type 2; n.a., not available; ns., not significant; * 65 FH subjects, but the results are pooled with non-FH subjects without statin treatment*

Adding bile acid sequestrant, cholestyramine, to the double treatment (statin + dietary plant stanol ester) for eight weeks, resulted in 67% decrease in LDL cholesterol from baseline (Gylling and Miettinen 2002c). In that study, all subjects reached the goal of LDL cholesterol <2.6 mmol/l with the triple treatment. To compare the additive efficacy of phytosterols and ezetimibe, combination of ezetimibe with statins resulted in ca. 15-25% additive reduction in LDL cholesterol in different hypercholesterolemic populations (Davidson et al. 2002, Gagné et al. 2002a). Thus, only a slight additive reduction seemed to be achieved by combining ezetimibe with statins compared with combining phytosterols. The triple treatment with statin, ezetimibe, and dietary phytosterols could offer a possibility to even more marked

reduction in serum cholesterol levels, but the efficacy of this triple treatment has not been evaluated yet.

2.6.6. Effects of dietary phytosterols on postprandial lipoproteins

Inhibition of cholesterol absorption might affect postprandial lipoproteins by decreasing the cholesterol flux from the intestine to the liver, thereby upregulating the LDL apoB receptors (Brown and Goldstein 1986). Inhibition of cholesterol absorption by plant stanol ester spread for two weeks resulted in diminished postprandial squalene and retinyl palmitate peak times and postprandial squalene concentrations and area under incremental curves (AUIC) (Relas et al. 2000), suggesting improved clearance of postprandial lipoproteins. However, AUIC for CM cholesterol appeared even to be increased. Thus, the improvement of postprandial lipoproteinemia was not due to acute inhibition of cholesterol absorption. The most probable explanation for improved clearance of TRL was the metabolic changes evoked by plant stanols. Accordingly, decreased pool of liver cholesterol leads to decreased VLDL synthesis (Gylling and Miettinen 1994b, Gylling and Miettinen 1996) and/or increased hepatic uptake of remnants by upregulated activity of LDL apoB receptors (Plat and Mensink 2002a).

The clearance of postprandial plant sterols has not been widely studied. A study by Relas et al. (2001b) showed that the clearance of sitosterol from CM-like emulsion after intravenous injection was more rapid than that of campesterol and squalene. Serum campesterol concentrations were lower after the addition of plant stanol esters to the test meal in an acute experiment (Relas et al. 2001a). It was speculated that this decrease could be associated with acute inhibition of cholesterol and other sterol absorption caused by stanol esters. However, little is known about the effects of more prolonged use of phytosterol ester spreads on the clearance of plant sterols.

2.7. Cholesterol synthesis and elimination

2.7.1. Cholesterol synthesis

Even though liver is the major organ responsible for cholesterol synthesis, most human cells are capable of synthesizing cholesterol (Dietschy et al. 1993). Cholesterol is synthesized from acetyl CoA via many biochemical steps (Figure 3), and the first rate-limiting step is the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) into mevalonic acid by HMG-CoA reductase. Mevalonate is transformed to squalene and further to lanosterol, which is then converted by different steps to cholesterol. The newly synthesized cholesterol is released into blood by VLDL, excreted into bile, or stored in an esterified form.

The amount of cholesterol in hepatocytes, either from endogenous synthesis or from diet transported by CM remnants to liver, is an important regulator of cholesterol synthesis. Accordingly, when the amount of cholesterol in liver cells increases, e.g., by increasing the dietary intake of cholesterol, cholesterol synthesis is downregulated, and vice versa (Brown and Goldstein 1986). In addition, the increased levels of hepatic cholesterol suppress the synthesis of LDL apoB receptors, therefore further preventing the accumulation of cholesterol in hepatocytes (Brown and Goldstein 1983). The rate of cholesterol synthesis in humans is around 1 g/day as measured by sterol balance technique, but this can be markedly increased in obesity (Miettinen et al. 1965, Grundy et al. 1965, Miettinen 1971). The rate of cholesterol synthesis is highest at night and lowest in the afternoon (Parker et al. 1982, Miettinen 1982).

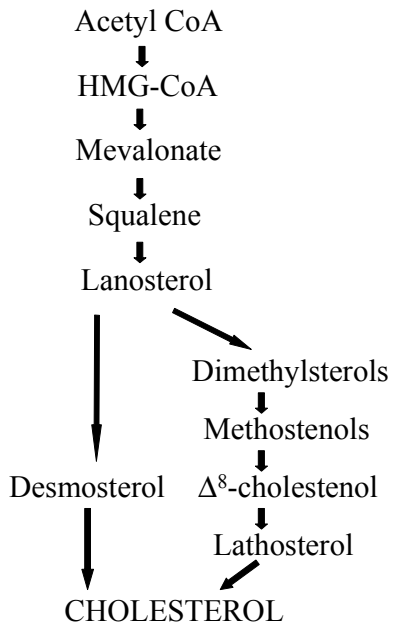

Figure 3. Simplified presentation of cholesterol synthesis.

2.7.1.1. Squalene and cholesterol precursor sterols

The process of cholesterol synthesis includes several intermediates (Figure 3), such as non-steroidal squalene and desmethyl sterols. The desmethyl sterols include e.g., Δ^8-cholestenol, desmosterol, and lathosterol, and these sterols are also called cholesterol precursor sterols. The serum levels of these cholesterol precursor sterols correlate positively with HMG-CoA reductase activity (Björkhem et al. 1987) and with cholesterol synthesis (Kempen et al. 1988, Miettinen et al. 1990b), and negatively with the cholesterol absorption efficiency and with serum plant sterols (Miettinen et al. 1990b). In addition, by inhibiting cholesterol synthesis, statins decrease the levels of serum cholesterol precursor sterols, especially lathosterol (Kempen et al. 1988, Uusitupa et al. 1992, Vanhanen and Miettinen 1992a, Vanhanen et al. 1992, Pfohl et

al. 1998). However, the serum ratios of squalene to cholesterol reflect cholesterol synthesis less consistently (Miettinen et al. 1990b), but may still reflect synthesis rate in acute experiments, such as immediately after LDL apheresis (Gylling et al. 1998). Thus, these cholesterol precursors can be measured by GLC in order to evaluate the changes in cholesterol synthesis.

Squalene is widely distributed in nature, e.g., in plant oils, and especially olive oil is rich in squalene (Liu et al. 1976). Thus, in addition to its endogenous synthesis, the normal daily human diet contains approximately 24 mg of squalene, which is readily absorbed (~65-80%) from the intestine (Miettinen and Vanhanen 1994b, Strandberg et al. 1990). The serum concentrations of squalene are approximately 35 μg/dl in healthy normolipidemic subjects (Liu et al. 1976). However, the serum concentrations of squalene may be even doubled in hypercholesterolemic subjects (Gylling and Miettinen 1994a, Nissinen et al. 2000). With respect to the cholesterol precursor sterols, the desmethyl sterols are almost exclusively endogenously synthesized in humans. The serum concentrations for Δ^8-cholestenol, desmosterol, and lathosterol were approximately 35 μg/dl, 140 μg/dl, and 335 μg/dl, respectively in a randomly selected Finnish population with moderately increased serum cholesterol levels (Nissinen et al. 2000). In the same population, the respective ratios to cholesterol were 14, 76, and 195 10^2 mmol/mol of cholesterol. In FH, the concentrations of all cholesterol precursors were higher than in the normal population (Gylling and Miettinen 1988).

Squalene and cholesterol precursor sterols are transported in lipoproteins in serum. Hepatic cholesterol synthesis increases the amount of cholesterol precursors in the liver leading to their release into the blood by VLDL. In fact, the newly synthesized squalene was found exclusively in VLDL (Goodman 1964). The highest ratio of squalene to cholesterol was also present in VLDL (Gylling and Miettinen 1994a). In addition, the ratio of lathosterol to cholesterol was highest in VLDL with and without statin treatment in type 2 diabetes (Miettinen and Gylling 2003) and in FH subjects with increased cholesterol synthesis due to ileal bypass (Koivisto and Miettinen 1988b). Statin treatment decreases the ratios of Δ^8-cholestenol, desmosterol, and lathosterol to cholesterol similarly in all lipoproteins in type 2 diabetes (Miettinen and Gylling 2003). Thus, it seems that changes in serum precursor sterols in all lipoproteins do reflect the changes occurring in cholesterol synthesis.

2.7.2. Cholesterol elimination

If one is to maintain a steady state in cholesterol metabolism, the human body must eliminate an equivalent amount of cholesterol as that synthesized by different tissues and absorbed from the intestine. The elimination of most of the cholesterol takes place in the liver, even though a small amount (~10%) of cholesterol is used in steroid hormone synthesis and desquamated by epithelial cells. HDL transports cholesterol from extrahepatic tissues to the liver, where the major pathway for elimination of cholesterol involves the secretion of cholesterol as such and bile acid synthesis by the hepatocytes. The enzymes, cholesterol 7α-hydroxylase and 27-hydroxylase play the major role in converting cholesterol into bile acids (Shefer et al. 1970, Björkhem 1992), mainly cholic acid and chenodeoxycholic acid. Bile acids

secreted from the liver pass through the intestine facilitating absorption of fats, sterols, and fat soluble vitamins. However, ~95% of bile acids are reabsorbed and returned to liver. Bile acids regulate their own synthesis by activating in conjunction with oxysterols, the nuclear hormone receptors, farnesoid X receptor and LXR (Chiang 2002). These receptors regulate the genes involved in bile acid synthesis, and thus an excess of bile acids can cause a feedback inhibition of bile acid synthesis.

2.7.3. Effects of dietary phytosterols on cholesterol synthesis and biliary secretion

Due to inhibition of cholesterol absorption by dietary phytosterols, cholesterol synthesis is compensatorily upregulated (Gylling and Miettinen 1994b, Gylling and Miettinen 1996, Gylling et al. 1997, Jones et al. 2000). In accordance with results from sterol balance studies, consumption of phytosterol esters increases the serum levels of all cholesterol precursor sterols, but most consistently that of lathosterol (Vanhanen et al. 1993, Gylling et al. 1997, Gylling et al. 1999, Hallikainen et al. 2000), also in FH subjects (Gylling et al. 1995, Vuorio et al. 2000) and in normolipidemic children (Tammi et al. 2002). The changes in serum squalene levels were minor and inconsistent (Vanhanen et al. 1993, Vanhanen et al. 1994, Gylling et al. 1995, Hallikainen et al. 2000). In addition, even with simultaneous inhibition of cholesterol synthesis by statins, consumption of plant stanol esters can increase the serum cholesterol precursor sterols in some (Vuorio et al. 2000, Gylling and Miettinen 2002a, Gylling and Miettinen 2002c, O'Neill 2003), but not in all studies (Vanhanen 1994). Furthermore, the study by O´Neill did not show any increments of lathosterol during consumption of plant stanol or sterol ester spread (O'Neill 2003). The above mentioned studies have evaluated the levels of the cholesterol precursors only in serum. Thus, it is not known whether the cholesterol precursors in different lipoproteins reflect the predicted change in cholesterol synthesis during consumption of phytosterol esters.

Contradictory results exist on the effects of phytosterols on bile acid metabolism. Several earlier studies have shown that consumption of free plant sterols led to an increase in fecal neutral, but not in acidic sterols, suggesting that bile acid synthesis was unchanged (Grundy et al. 1969, Kudchodkar et al. 1976, Becker et al. 1993). Similar results were obtained with plant stanol esters (Miettinen and Vanhanen 1994a, Gylling and Miettinen 1994b, Gylling et al. 1997, Miettinen et al. 2000c). However, Becker et al. (1993) reported that consumption of plant stanols increased the fecal bile acid excretion in children. The authors speculated that this increase could be either due to upregulated cholesterol synthesis or due to decreased serum plant sterol levels, which could be associated with increased bile acid synthesis. In fact, high plasma and liver sitosterol levels in phytosterolemic subjects inhibited 7α-hydroxylase, a rate limiting enzyme in bile acid synthesis, but did not change the total hepatic bile acid pool (Shefer et al. 1994). However, fecal bile acid excretion was decreased by 21% during consumption of plant sterol esters (Weststrate et al. 1999). Further, consumption of plant sterol esters decreased the concentrations of 7α-hydroxy cholestenone, an index of cholesterol 7α-hydroxylase activity (O'Neill et al. 2002, O'Neill 2003). There was no change in 7α-hydroxy cholestenone levels during consumption of plant stanol ester spread. These results suggest that serum plant sterol

levels may regulate the bile acid synthesis, and accordingly a high dietary intake of plant sterols, but not of plant stanols, may decrease bile acid synthesis, which was suggested to be one reason for the rebound effect in total and LDL cholesterol levels (O'Neill et al. 2002, O'Neill 2003).

2.8. Familial hypercholesterolemia

FH is a dominantly inherited metabolic disorder that affects around one in 500 individuals in its heterozygous form (Goldstein et al. 2001). This disease is caused by a mutation in a gene coding for the LDL apoB receptor, leading to impaired uptake of LDL particles in all tissues, especially in liver. Accordingly, LDL cholesterol concentrations are increased approximately two- to three-fold in heterozygotes. Individuals with two mutated alleles of the LDL apoB receptor gene (FH homozygotes), with prevalence of 1:1 000 000, have no or only minor activity of LDL apoB receptors, leading to up to four-fold concentrations of serum LDL cholesterol. In addition, serum HDL cholesterol levels are slightly lower in both heterozygous and homozygous subjects than in normal subjects, but serum TG levels are normal.

2.8.1. Clinical features and diagnosis

In FH subjects, the excess cholesterol is accumulated in tissues causing tendon xanthomas, skin xanthelasmas, corneal arcus and atherosclerosis leading to CHD already at young age (Goldstein et al. 2001). In fact, atherosclerotic lesions have been obtained in children with FH already at ages 3-19 years (Tonstad et al. 1996, Virkola et al. 1997). Many genetic and environmental factors, including gender, smoking, the presence of hypertension, and hypertriglyceridemia influence the expression of CHD (Hill et al. 1991), explaining the wide variations in the clinical phenotype in FH subjects. The diagnosis of heterozygous FH in adults and children has traditionally been made according to typical clinical traits, including high concentrations of total (> 7.5-8.5 mmol/l) and LDL cholesterol, a family history of elevated concentrations of LDL cholesterol and premature atherosclerosis, and presence of tendon xanthomas (Goldstein et al. 2001). Nowadays, especially in populations with a high degree of genetic homogeneity, the diagnosis of FH can be made by detection of the specific LDL apoB receptor mutation using DNA extracted from white blood cells and polymerase chain reaction (PCR)-based techniques (Goldstein et al. 2001), or quantifying LDL apoB receptor function from cultured fibroblasts (Cuthbert et al. 1986).

2.8.2. Genetics

The gene for LDL apoB receptor is located on the short arm of chromosome 19 (Francke et al. 1984). Over 900 different LDL apoB receptor mutations have been identified (www.ucl.ac.uk/fh/) ranging from single nucleotide substitutions to large deletions of the gene. As a consequence, different mutations result in proteins with different structures and functions. Accordingly, LDL apoB receptor mutations can be divided into five classes based on their function in producing and transporting the protein, binding of LDL, internalizing of LDL, and recycling the protein back to the

cell surface (Goldstein et al. 2001). The mutation phenotype may affect the clinical course of the disease and e.g., the response to hypolipidemic medication (Leitersdorf et al. 1993).

In Finland, ~25 different mutations of the LDL apoB receptor gene have been identified (Vuorio et al. 2001). However, owing to a founder effect, two major mutations, FH-Helsinki (FH-Hki) (Aalto-Setälä et al. 1989) and FH-North Karelia (FH-NK) (Koivisto et al. 1992), causing deletion of the LDL apoB receptor gene, accompanied with two point-mutations FH-Turku and FH-Pori (Koivisto et al. 1995) account for about three fourths of all the FH cases identified in Finland.

2.8.3. Cholesterol metabolism in familial hypercholesterolemia

2.8.3.1. Cholesterol absorption

Cholesterol absorption in adult subjects with heterozygous FH (Connor and Lin 1974, Sodhi et al. 1980, Gylling and Miettinen 1989) and in children with homozygous FH FH (Carter et al. 1979) has been shown to be similar to normolipidemic subjects. In contrast to normolipidemic subjects, the serum lipid levels are not correlated with cholesterol absorption in FH (Gylling and Miettinen 1989). However, as in the normal population (Kesäniemi and Miettinen 1987, Miettinen and Kesäniemi 1989), the absorption of cholesterol is negatively correlated with cholesterol synthesis in FH (Gylling and Miettinen 1989). Accordingly, efficient absorption of cholesterol will result in lower synthesis of cholesterol also in FH.

2.8.3.2. Cholesterol and bile acid synthesis

Despite impaired cellular uptake of LDL by LDL apoB receptors in FH, hepatic cholesterol levels are elevated due to high serum concentrations of cholesterol and an appreciable amount of LDL taken up by a non-receptor mediated pathway (Thompson et al. 1996). This increase in hepatic cholesterol levels is sufficient to maintain the cholesterol synthesis at low normal limits in heterozygous and homozygous FH adults (Bilheimer et al. 1979, Goldstein et al. 2001) or adolescents (Carter et al. 1979) and in heterozygous children (Martin and Nestel 1979), even though wide variations between families may exist (Miettinen 1984). However, according to several studies, young FH homozygotes, aged under 10 years, may exhibit an overproduction of cholesterol (Bilheimer et al. 1975, Schwarz et al. 1979, Miettinen 1984) compared with heterozygous or normolipidemic subjects. As in the normal population, the serum levels of cholesterol precursor sterols reflect cholesterol synthesis in FH (Gylling and Miettinen 1988), and thus may be used as markers of cholesterol synthesis.

The rate of bile acid synthesis may vary between families (Miettinen 1984), but mostly subnormal (Miettinen 1978, Simonen and Miettinen 1987) or normal (Moutafis et al. 1977, Zavoral et al. 1982) rates have been obtained.

Even though in general overall cholesterol synthesis is normal, the increased hepatic cholesterol increases the production of VLDL apoB (Cummings et al. 1995), which is

then converted to IDL. Due to the defect in LDL apoB receptor, IDL is poorly taken up by the liver, and thus is converted to LDL (Brown and Goldstein 1986). Accordingly, in addition to reduced degradation, FH is accompanied with overproduction of LDL.

2.8.3.3. Postprandial lipoproteins

In FH, the impaired or total lack of activity of the LDL apoB receptor suggests that the clearance of TRL might be decreased. However, the results from earlier studies are controversial. Four studies showed normal clearance of TRL both in heterozygous (Weintraub et al. 1987, Eriksson et al. 1991, Watts et al. 2001) and homozygous FH (Rubinsztein et al. 1990, Watts et al. 2001) subjects, while other studies have reported delayed CM remnant clearance in both heterozygous and homozygous FH (Cabezas et al. 1998, Mamo et al. 1998, Twickler et al. 2000). In addition, fasting concentrations of apoB-48 and RLP cholesterol were higher in FH subjects compared with normolipidemic subjects (Dane-Stewart et al. 2001, de Sauvage Nolting et al. 2002). The discrepancy in results of FH subjects reflects the variations in the clinical phenotype of FH, and suggests that liver receptors other than LDL apoB receptor may play an important role in TRL clearance. However, as in most studies in non-FH population (Karpe 2002), statins improve the clearance of postprandial lipoproteins (Twickler et al. 2000, Dane-Stewart et al. 2002) and reduce the RLP cholesterol levels (de Sauvage Nolting et al. 2002) also in FH. The fact that statins upregulate the expression of LDL apoB receptors (Brown and Goldstein 1986, Reihnér et al. 1990) indicates that LDL apoB receptor must, however, have a role in the clearance of TRL also in FH.

2.8.4. Treatment

Heterozygotes

Decreased serum LDL cholesterol levels in FH may be achieved either by increasing the uptake of LDL particles via upregulation of LDL apoB receptors and/or a decrease in LDL production. Dietary interventions with reduced amount of dietary cholesterol and saturated fatty acids are not sufficiently effective in lowering LDL cholesterol in FH to the recommended levels, but must be a part of the lipid-lowering therapy, in combination with pharmacological agents. Resins act by inhibiting the absorption of bile acids, thus increasing the conversion of hepatic cholesterol into bile acids and promoting a compensatory rise in cholesterol synthesis and upregulation of LDL apoB receptors. Resins lower LDL cholesterol levels by ~15-30% (Moutafis et al. 1977, Miettinen and Lempinen 1977). On the other hand, statins inhibit cholesterol synthesis by blocking the HMG-CoA reductase activity, decreasing the amount of hepatic cholesterol, and thereby upregulating the LDL apoB receptors (Brown and Goldstein 1986, Reihnér et al. 1990, Bergström et al. 1998). This latter factor and the reduced production of VLDL by statins (Isusi et al. 2000) lead to an approximate mean reduction of 39% in LDL cholesterol in heterozygous FH with different statins (Hopkins 2003). However, according to a recent study by Smilde and colleagues (2001), at least 45% reduction in LDL cholesterol is required to achieve a regression in intima media thickness in heterozygous FH. Thus, combination of statins with

resins (Uusitupa et al. 1992, Illingworth 1993), fibrates (Muratti et al. 1994), ezetimibe (Davidson et al. 2002), or plant stanol or sterol esters (Vuorio et al. 2000, O'Neill et al. 2002) to achieve an additional 5-25% reduction in LDL cholesterol is recommended. In addition, up to 67% reduction in LDL cholesterol was achieved by triple treatment with statin, plant stanol esters, and cholestyramine in FH subjects (Gylling and Miettinen 2002c).

Homozygotes

Generally, FH homozygotes are resistant to treatments that are effective in FH heterozygotes, because they produce only a few functional LDL apoB receptors (Goldstein et al. 2001). Accordingly, at the very best, only ~30% reduction in LDL cholesterol levels has been obtained by monotherapy with a high statin dose (Raal et al. 2000) or in combination with ezetimibe (Gagné et al. 2002b). Additional treatment options for the homozygote FH subjects in the past included ileal by-pass or portacaval anastomosis, but more recent treatment options include liver transplantation and LDL apheresis. In addition, FH was suggested to be an excellent candidate for gene therapy. However, LDL apoB receptor gene replacement studies have been restricted mostly to animal studies (Hopkins 2003). One small pilot study in humans showed no or only a modest reduction in LDL cholesterol (Grossman et al. 1995). Thus, gene therapy will require major advances and improvements in the future before it can become a potential treatment option for FH subjects.

The different treatment procedures alter cholesterol metabolism in different ways. Ileal by-pass operation increases intestinal loss of bile acids and is accompanied by increased cholesterol synthesis (Miettinen 1979), while portacaval anastomosis causes a decrease in cholesterol synthesis resulting in 25% to 50% reduction in LDL cholesterol (Starzl et al. 1973, Bilheimer et al. 1975). Liver transplantation provides an external source of LDL apoB receptors and causes a dramatic fall in LDL cholesterol levels and resolution of xanthomas (Bilheimer 1989, Goldstein et al. 2001). LDL apheresis provides a method to remove excess LDL cholesterol from plasma. Repeated fortnightly procedures are accompanied with ~50% reduction in LDL cholesterol and arrest of progression of CHD (Kroon et al. 1996). Chronic LDL apheresis treatment causes no or even slight decrease in cholesterol synthesis (Pfohl et al. 1994, Gylling et al. 1998), whereas a transient increase in cholesterol synthesis was observed in FH homozygotes after LDL apheresis (Pfohl et al. 1994, Gylling et al. 1998). Despite the fact that there may be even more profound upregulation of cholesterol synthesis in these subjects with statin treatment than if they are left untreated, statins do effectively reduce LDL cholesterol and cholesterol synthesis in long-term use and thus are recommended in combination with LDL apheresis (Yamamoto et al. 2000, Goldammer et al. 2002).

Children

Since the early signs of atherosclerosis can be detected already in childhood in FH (Tonstad et al. 1996, Virkola et al. 1997), treatment of increased LDL cholesterol levels shoud be started as early as possible. Initially, lifestyle advice is recommended to reduce serum cholesterol levels in FH children. Resins are first-line drugs for

treatment of children, since they are not absorbed from the intestine and are safe and effective achieving ~25% reduction in LDL cholesterol (Marks et al. 2003). However, the compliance for resin therapy is poor, with only ~50% of patients remaining on the treatment. Better compliance achieving ~15% decrease in LDL cholesterol has been obtained by plant stanol or sterol esters (Gylling et al. 1995, Vuorio et al. 2000, Amundsen et al. 2002, de Jongh et al. 2003). However, no effect on endothelial function could be obtained during consumption of plant sterol esters (de Jongh et al. 2003). Statins have been shown to be effective and safe in short- (Knipscheer et al. 1996, Lambert et al. 1996) and long-term studies resulting in 27-40% decreases in LDL cholesterol with different statins (Stein et al. 1999, de Jongh et al. 2002b, McCrindle et al. 2003). Additionally, 28-week treatment with simvastatin improved endothelial function in children with FH (de Jongh et al. 2002a).

3. AIMS OF THE STUDY

Dietary phytosterols lower serum cholesterol levels by inhibiting cholesterol absorption. In addition, dietary phytosterols have several effects on sterol metabolism. The consumption of plant sterol and stanol esters alter differently the serum plant sterol levels in adults, whereas the cholesterol synthesis markers are increased by both phytosterol products. However, nothing is known about the effects of plant sterol esters on the accumulation of plant sterols in blood in children and in homozygous FH subjects. In addition, the effects of combination of cholesterol synthesis inhibition by statin treatment and cholesterol absorption inhibition especially by plant sterol esters on serum, lipoprotein, and red cell non-cholesterol sterol values is limited. Further, only one study has evaluated the effects of plant stanols on postprandial lipoproteinemia in non-FH subjects. Accordingly, the effects of dietary phytosterols in statin-treated FH on clearance of postprandial lipoproteins have not been studied. Finally, the effects of disturbed cholesterol metabolism in FH with and without statin treatment, and the effects of phytosterol consumption on distribution of non-cholesterol sterols are not known.

Therefore, the aims of the present study were to investigate

- distribution of non-cholesterol sterols in different serum lipoproteins in children with FH and in normolipidemic children

- effects of dietary plant stanol and sterol esters on serum and red cell plant sterol and other non-cholesterol sterol levels in hypercholesterolemic children

- effects of inhibiting cholesterol absorption by plant stanol and sterol esters on serum, lipoprotein, and red cell plant sterol and other non-cholesterol sterol levels in heterozygous FH subjects during inhibition of cholesterol synthesis by statin treatment

- effects of inhibiting cholesterol absorption by plant stanol and sterol esters on serum and lipoprotein plant sterol and other non-cholesterol sterol levels in a subject with homozygous FH on statin and LDL apheresis treatment

- effects of inhibition of cholesterol absorption and alteration of serum plant sterol levels by dietary plant stanol and sterol esters on postprandial lipoproteinemia in statin-treated heterozygous FH subjects

4. SUBJECTS AND METHODS

4.1. Subjects and study design

A total of 67 children (32 with heterozygous FH, 6 hypercholesterolemic, and 29 non-affected children) were recruited by personal letters according to patient data from Outpatient Clinics of the Hospital for Children and Adolescents, University of Helsinki. In addition, one homozygous FH subject and 20 adult patients with heterozygous FH were recruited from the Meilahti Hospital, University of Helsinki (Table 2). Diabetes, kidney, liver, and thyroid diseases were exclusion criteria in all of the subjects. With the exception of the intervention periods, the subjects continued their normal habitual diet throughout the studies. Possible use of plant stanol or sterol ester spreads was terminated at least three weeks prior to the studies. The dose of plant stanol or sterol ester spreads in Studies II-V was 25 g/day (2 g of free plant stanols and sterols daily). Weighing the returned unwashed spread containers provided an approximation of the amount of consumed spreads in Studies II-V. The prescribed medication was continued unchanged during the studies. All of the subjects or parents for children under 12 years gave their informed consent. The study protocols had been accepted by Ethics Committee of the Hospital for Children and Adolescents or the Department of Medicine, University of Helsinki.

Table 2. Baseline characteristics of the subjects

Variable	***Study I***		***Study II***	***Study III***	***Study IV***		***Study V***
	FH	*non-FH*	*Hyperchol.*	*FH+statin*	*Family Study*		*FH+statin*
	Children		*Children*	*Adults*	*HeZ*	*HoZ*	*Adults*
n	*18*	*29*	*23*	*18*	*3*		*5*
sex (m/f)	*10/8*	*14/15*	*10/13*	*6/12*	*1/2*		*1/4*
FH subjects n	*18*	*0*	*17*	*18*	*3*		*5*
age (years)[1]	*11±0.8*	*11±0.6*	*6.8±0.5*	*48±2*	*44±7*		*51±4*

[1]*Mean±SEM; FH, familial hypercholesterolemia; HeZ, heterozygous FH; HoZ, homozygous FH; Hyperchol., hypercholesterolemic*

Study I

The study group consisted of 47 children aged 5 to 16 years. All of the subjects had a first or second degree relative with clinically or genetically documented FH, or were themselves patients at the Outpatient Lipid Clinics of the Hospital for Children and Adolescents, University of Helsinki. The subjects were divided into FH and non-FH subjects by DNA analysis characterizing the four most common Finnish LDL apoB receptor mutations (Aalto-Setälä et al. 1989, Koivisto et al. 1992, Koivisto et al. 1995). Serum total cholesterol was < 5.0 mmol/l in non-FH group. The subjects visited the Outpatient Clinics once, when routine clinical status was assessed. The blood sample was obtained after at least 4 hours' fasting.

Study II

The study population comprised 23 prepubertal children aged 2 to 9 years. The inclusion criteria included total cholesterol > 5.0 mmol/l and serum TG < 2.0 mmol/l. None of the children were taking any hypolipidemic medication. Of the 23 children, 16 had FH according to prior DNA analysis, and one child with lipid levels appropriate to FH, and with a positive family history of FH, was included in the FH group. The remaining six subjects were negative for LDL apoB receptor mutations and did not have a family history of FH.

The study had a double-blind, randomized, cross-over design. It consisted of two 5-week intervention periods with plant stanol and sterol ester spreads, and with a 5-week wash-out period between the interventions.

The subjects visited the Outpatient Clinics four times, at the beginning and end of every period. The routine physical examination was performed on the first and last visits. The blood samples were taken after an overnight fast on every visit. Due to small blood sample size, only plasma samples were obtained.

Study III and V

The study group consisted of 18 (Study III) and five (Study V) adult heterozygous FH subjects. All subjects had used the same statin dose for several years. Thus, the baseline measurement was made during statin treatment, and is called ‘baseline’ in the following sections. As equivalent dose of atorvastatin, the mean statin dose used in Study III was 40 mg/day. In addition, two subjects used bile acid sequestrants in Study III.

The studies had a double-blind, randomized, cross-over design. The subjects were advised to consume low fat plant stanol and sterol ester spreads in a random order for two four-week periods without any wash-out period. The subjects visited the Outpatient Clinics at the beginning and end of the periods, when the physical examination was performed and the fasting blood samples were drawn a few days apart. The mean of the two samples in all serum determinations was used in statistical calculations. The Intralipid fat tolerance test was performed three times (Study V), at baseline and at the end of the two intervention periods.

Study IV

The study population comprised two heterozygous FH parents (LDL apoB receptor mutation FH-Hki for the one parent and FH-NK for the other parent) and their 29-year-old offspring with homozygous FH with FH-Hki and FH-NK mutations. The diagnosis for the homozygous subject was made at the age of 4 years. The subject had undergone portacaval shunt, ileal bypass, aortic valve, and coronary artery operations. Statin therapy had been started in the late 1980’s. LDL-apheresis was started in 1990, and has continued since that date. The prescribed medication, atorvastatin 80 mg/day, as well as the LDL apheresis treatments continued as before the study. The parents

were not consuming any basal medications nor did they exhibit clinical signs of CHD.

In the homozygous subject, LDL apheresis treatments were scheduled to occur once per fortnight. Due to patient-related reasons the intervals were occasionally extended up to three or even four weeks. LDL apheresis was performed with Liposorber MA-01 (Kaneka Corporation, Osaka, Japan) with two columns of dextran sulphate cellulose beads as the adsorbent. Four liters of plasma were treated during every apheresis using heparin as the anticoagulant. The subject was advised to start low fat plant sterol ester spread for 73 days (3 apheresis treatment intervals) followed by low fat plant stanol ester spread for 157 days (9 apheresis treatment intervals). Pre-apheresis blood samples were drawn after an overnight fast. Three weeks after the discontinuation of the stanol ester consumption, a blood sample was taken during the home diet.

After a routine physical examination, the parents were advised to consume in double blind and in random order low fat plant stanol and sterol ester spreads both for 4 weeks. The blood samples were taken after an overnight fast before and after each period, and after 4 weeks of the intervention on the home diet.

The spreads

In Studies II and IV, the plant stanol and plant sterol ester spreads were similar as the spreads in the markets (Light Benecol, Raisio Benecol Ltd, Raisio, Finland, 32% of fat and Becel pro.activ, Unilever BestFoods, Purfleet, UK, 35% of fat). Accordingly, the fatty acid compositions of the plant stanol ester and sterol ester spreads were 16% and 25% of saturated fatty acids, 56% and 25% of monounsaturated fatty acids, and 28% and 50% of polyunsaturated fatty acids, respectively. Both spreads contained 6 mg/100g and 66 mg/100g of vitamin E, respectively. The plant stanol ester spread contained 1.8% sitosterol, 1.5% campesterol, 0% stigmasterol, 0% avenasterol, 0.05% cholesterol, 66.8% sitostanol, and 29.8% campestanol. The respective values for plant sterol ester spread were 51.5%, 24.3%, 20.5%, 1.3%, 0.4%, 1.2%, and 0.6%.

In Studies III and V, both spreads contained 40% of fat and the fatty acid distribution of the spreads were similar; 22% of saturated fatty acids, 34% of monounsaturated fatty acids, and 44% of polyunsaturated fatty acids. The plant stanol ester spread contained 1.6% sitosterol, 2.0% campesterol, 0% stigmasterol, 0% avenasterol, 0% cholesterol, 65.6% sitostanol, and 28.8% campestanol. The respective values for plant sterol ester spread were 45.3%, 26.1%, 14.5%, 3.5%, 0.4%, 0%, and 1.2%. Both spreads contained 17 mg/100 g of vitamin E. The amounts of vitamin A (900 µg/100g) and vitamin D (7.5 µg/100g) were similar in all of the spreads.

4.2. Methods

4.2.1. Clinical examination

A routine clinical examination of the subjects included measurement of height and weight, puberty staging (Study I) (Tanner 1969), blood pressure measurement,

auscultation of heart and lungs, palpation of tendons, and inspection of the presence of xanthelasmas and corneal arcus.

4.2.2. DNA diagnosis

DNA was isolated from the frozen EDTA-anticoagulated blood samples using a prodedure modified from Kunkel et al. (1977) in Study I. PCR (Koivisto et al. 1993) was used to analyze the four most common mutations (FH-Hki, FH-NK, FH-Turku, and FH-Pori) (Aalto-Setälä et al. 1989, Koivisto et al. 1992, Koivisto et al. 1995) of the LDL apoB receptor gene in Finland.

4.2.3. Lipoprotein separation

CM were separated in Studies III and V by a 30-min ultracentrifugation (18000 RPM) in a fixed-angle Type 50 Ti rotor (Beckman, Palo Alto, CA, USA) with carefully overlayered 1.006 g/ml NaCl solution. In Study I, other lipoproteins were separated by ultracentrifugation in a fixed-angle Type 50.4 Ti rotor (Beckman, Palo Alto, CA, USA) (35000 RPM, 18-20 hours) according to their densities: VLDL, < 1.006 g/ml; IDL, 1.006-1.019 g/ml; LDL, 1.019-1.063 g/ml and HDL, 1.063-1.210 g/ml (Havel et al. 1955, Warnick and Alberts 1982). In Studies III-V, the lipoproteins were separated only into VLDL and VLDL infranatant (1.006-1.210 g/ml). From the infranatant, HDL was separated by precipitation of apoB containing lipoproteins with phosphotungstic acid and magnesium (Roche, Basel, Switzerland). The sediment included LDL and IDL, but will be designated as LDL in the following section.

4.2.4. Serum and lipoprotein lipids

In Study I, serum and lipoprotein total and free cholesterol, phospholipids, and serum TG as well as plasma total, free, and HDL cholesterol and TG in Study II and total, HDL, and VLDL cholesterol in Study IV were analyzed by enzymatic color reactions with commercial kits (total and lipoprotein cholesterol: CHOD-PAP; TG: GPO-PAP, Roche, Basel, Switzerland; phospholipids: phospholipase D choline-oxidase PAP, Wako Chemicals, Neuss, Germany; free cholesterol: CHOD-PAP, Boehringer-Mannheim, Mannheim, Germany) by using a semiautomatic COBAS Mira analyzer (Roche, Basel, Switzerland). Cholesterol and TG were analysed from the six subjects in Study III in all lipoproteins and in Study V in CM and VLDL by commercial kits (cholesterol: CHOD-PAP, TG: GPO-PAP, ABX-Diagnostics, Parc Euromédecine, France). In Studies III and V, the serum total and HDL cholesterol and serum TG were analyzed by the routine methods of our hospital. LDL cholesterol was calculated by Friedewald (Friedewald et al. 1972) (Studies II, III, and V) or by subtracting the levels of VLDL and HDL cholesterol from serum cholesterol (Study IV).

4.2.5. Squalene and non-cholesterol sterols

The red cells were washed with 0.9% sodium chloride three times and centrifuged at 3000 RPM for 5 minutes before the analyses.

Serum, red cell, and lipoprotein cholesterol, squalene, and non-cholesterol sterols (cholestanol, Δ^8-cholestenol, desmosterol, lathosterol, campesterol, campestanol,

sitosterol, avenasterol, and sitostanol) were quantified by GLC on a 50-m-long SE-30 capillary column (Ultra-1 or Ultra-2; Hewlett-Packard, Wilmington, DE, USA) (Miettinen and Koivisto 1983, Miettinen 1988). Briefly, after addition of 100 µl of 5α-cholestane as an internal standard, 200-1000 µl of serum, red cell, or lipoprotein samples were saponified with 99% ethanol and potassium-hydroxide (10M) (9:1, vol:vol) for 1.5 hour in 80 °C. The nonsaponified lipids were extracted by hexane once or twice in case of the smaller fractions. The hexane was evaporated under a stream of nitrogen and followed by silylation of lipids by trimethylsilylate to bind free O^- groups of sterols before the GLC run. Cholesterol, squalene, and non-cholesterol sterols were also measured from the spreads and the Intralipid-squalene mixture.

To eliminate the effects of different cholesterol levels in serum, red cells, and lipoproteins, the values of squalene and non-cholesterol sterols are expressed as ratios to cholesterol in terms of 10^2x mmol/mol of cholesterol. In most studies, the concentrations of squalene and non-cholesterol sterols are also given. For simplicity, squalene data are given under the topic non-cholesterol sterols.

4.2.6. Fat tolerance test with Intralipid-squalene

Intralipid emulsion added with squalene was performed by dissolving liquid 98-100% squalene (Sigma, St. Louis, MO, USA) into commercially available Intralipid 200 mg/ml fat emulsion (Pharmacia, Stockholm, Sweden). To ensure the sterility of squalene, it was first filtered with a sterile Millex-GV 0.22 µm pore size filter unit (Millipore SA, Molsheim, France), after which 80 mg (100 µl) of squalene was dissolved in 900 µl of 99.5% ethanol. The squalene-ethanol solution was added to 100 ml of Intralipid. Accordingly, the emulsion contained squalene at 69.8±4.8 mg/dl, cholesterol at 25.9 mg/dl, TG at 20 g/dl, campesterol at 6.2 mg/dl, and sitosterol at 20.8 mg/dl.

After taking the fasting blood sample in the morning, 30 ml of the Intralipid+squalene emulsion was injected as a bolus into a forearm vein over 1-2 min. The blood samples were collected from the opposite forearm via an intravenous cannula 10, 20, 40, 60, 120, and 180 min after the injection.

In the calculations, TG, squalene, and plant sterol levels were provided as incremental concentrations, calculated by subtracting the basal value from the value at the respective time point. AUIC values were calculated by the trapezoid method.

4.2.7. Other measurements

The apoE phenotype was determined electrophoretically by isoelectric focusing from serum samples (Havekes et al. 1987) in Study I. Subjects with phenotypes apoE-2/2, apoE-3/2, and apoE-4/2 were combined and called apoE-2 group, those with apoE-3/3 were called apoE-3 group, and subjects with apoE-3/4 or apoE-4/4 were called apoE-4 group.

Blood glucose was measured with a commercial kit using glucose dehydrogenase method (Roche, Basel, Switzerland), serum insulin was analyzed by radioimmuno-assay (Pharmacia & Upjohn, Uppsala, Sweden), and sex hormone globulin by commercial kits (Wallac, Turku, Finland).

4.2.8. Statistics

All the statistical analyses were performed using Microsoft Excel version 6.0 and the Number Cruncher Statistical System (NCSS) (NCSS, 2000, Kaysville, Utah, U.S.A.). Continuous values are given as mean ± standard error of the mean (SEM). Calculations were made according to intention-to-treat principle. The carry-over effect was controlled by analysis of variance (ANOVA) for repeated measurements in Study II, and the effect of the treatment sequence was evaluated by two-way ANOVA with the treatment period as the repeated factor and the sequence of treatment as the ”group” factor in Studies III and V. The statistical significances between the groups were calculated by one-way ANOVA, two-sided t test, or χ^2 test. Paired t test was used to evaluate the treatment effects in Studies II, III, and V. Half-lives in Study V were calculated by nonlinear least-square analysis from the absolute concentrations. If a variable was skewed, either logarithmic transformation was made or data were analyzed by non-parametric tests. The correlation coefficients were calculated by using Pearson’s product moment or Spearman’s rank correlation test in appropriate cases. A p value < 0.05 was considered significant.

Analysis for appropriate number of subjects was performed in Studies II and III. Thus, the calculations were made with $\alpha=0.05$ and $\beta=0.20$ and a minimal detectable difference of 0.40 mmol/l for LDL cholesterol.

5. RESULTS

5.1. Clinical characteristics and compliance

Age, sex, height, weight, body mass index, and blood pressure were similar in the children with or without FH. No clinically recognizable signs of lipid accumulation, such as tendon xanthomas, were detected in FH children. 21 children in Study I had reached puberty, but the children in Study II were in prepuberty.

In all heterozygous adults in Study III and in the homozygous subject in Study IV, tendon xanthomas, xanthelasmas, and corneal arcus were detected. The homozygous and four heterozygous FH subjects had a diagnosis of CHD.

The compliance in the intervention studies was good. Only one child in Study II did not complete the two interventions. According to weight of the returned spread containers, the adults consumed up to 100% and children from 80% to 85% of the scheduled plant stanol and sterol ester spreads, respectively. No side effects were reported. There was no change in body weight during 15-week consumption of phytosterol ester spreads in children (Study II). The weight and blood pressure of the adult subjects consuming plant stanol and sterol ester spreads remained constant during the interventions (Studies III and IV).

5.2. Baseline values

We wanted to evaluate the non-cholesterol sterols in serum and their distribution in different lipoproteins in FH with marked hypercholesterolemia and in their non-affected controls. Further, we also wanted to determine whether additional data from cholesterol metabolism can be obtained by analyzing the non-cholesterol sterols from different lipoproteins. Study I concentrated on baseline values, but we collected baseline data also from intervention studies, in order to obtain information on factors affecting the response to phytosterol ester treatment in the present study population.

5.2.1. Serum and lipoprotein lipids

The baseline lipid values of Studies II-IV and the lipids of Study I are shown in Table 3. In Study IV a mean of two baseline measurements was calculated for two heterozygous subjects not receiving statin treatment. Serum and LDL cholesterol levels were higher in children with FH than in non-affected normolipidemic children, whereas HDL cholesterol was similar in the two groups. Despite the long-term statin treatment, the total and LDL cholesterol levels in FH adults (Study III) were still above 5.0 mmol/l and 3.0 mmol/l, respectively, but lower than in FH adult subjects without statin treatment (Study IV). LDL cholesterol was only insignificantly correlated with the statin dose in Study III ($r = -0.333$). Serum total and LDL cholesterol values were the same during the home diet in heterozygous parents without any lipid-lowering medication and in the homozygous offspring on statin treatment and repeated LDL apheresis.

Gender or the type of LDL apoB receptor mutations did not affect the serum lipid levels. In children without FH, but not in those with FH, serum total and LDL cholesterol levels were higher in subjects with apoE-4 phenotype than in subjects with combined apoE-2 and apoE-3 phenotypes (Study I). The esterification percentages of serum (~70%), VLDL (60-70%), and LDL (~70%) were similar in children with and without FH, whereas the esterification percentages of HDL (83% vs. 81%) and IDL (69% vs. 60%) were higher in FH than in non-FH children (Table 2, in Study I).

Table 3. Baseline lipids (mmol/l) of the study subjects

Variable	***Study I***		***Study II***	***Study III*** [a]	***Study IV***	
	FH	*non-FH*	*Hyperchol.*	*FH + statin*	*Family study*	
	Children		*Children*	*Adults*	*HeZ*	*HoZ*
	n=18	*n=29*	*n=23*	*n=18*	*n=2* [b]	*n=1*
Serum Chol	*7.5±0.3**	*4.2±0.1*	*6.8 ±0.3*	*6.3±0.2*	*10.4, 9.6*	*9.4*
LDL Chol	*5.5±0.3**	*2.2±0.1*	*5.2±0.3*	*4.5±0.2*	*8.5, 8.1*	*8.2*
HDL Chol	*1.2±0.1*	*1.3±0.04*	*1.1±0.1*	*1.3±0.1*	*1.3, 1.3*	*1.1*
VLDL Chol	*0.2±0.03*	*0.2±0.03*	*n.a.*	*0.3±0.1* [c]	*0.5, 0.2*	*0.01*
IDL Chol	*0.1±0.02*	*0.1±0.01*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
CM Chol	*n.a.*	*n.a.*	*n.a.*	*3.0±0.4*	*n.a.*	*n.a.*
Serum TG	*0.8±0.1*	*0.9±0.1*	*1.0±0.1*	*1.2±0.1*	*1.6, 0.9*	*0.7*

*Mean±SEM, except in Study IV; Chol, cholesterol; FH, familial hypercholesterolemia; HeZ, heterozygous FH; HoZ, homozygous FH; Hyperchol., hypercholesterolemic; n.a., not available; [a] subjects from study V are included, [b] mean of two measurements/person, [c] measured by GLC, * p<0.05 vs. non-FH children*

5.2.2. Serum non-cholesterol sterols

At baseline, serum concentrations of most non-cholesterol sterols were higher in children with FH than in non-affected children (Table 4). In the homozygous subject, the concentrations of serum cholesterol precursor sterols were at the upper limits of heterozygous parents. The levels of plant sterols were mostly similar in both children and adult study populations, even though the concentration of sitosterol was lower in heterozygous adults not treated with statins compared with those on statin treatment. The serum levels of plant sterols and cholestanol, the absorption markers, were markedly higher in the homozygous subject than in the heterozygous parents. The highest plant stanol values were obtained in statin-treated FH subjects and in the homozygous FH subject.

Table 4. Baseline cholesterol (mg/dl), squalene, and non-cholesterol sterol (μg/dl) concentrations

Variable	***Study I***		***Study II***	***Study III***[a]	***Study IV***	
	FH	*non-FH*	*Hyperchol.*	*FH with statin*	*Family Study*	
	Children		*Children*	*Adults*	*HeZ*	*HoZ*
	n=18	*n=29*	*n=23*	*n=18*	*n=2*[b]	*n=1*
cholesterol	*267±11**	*152±4*	*248±9*	*212±8*	*375, 346*	*333*
squalene	*63±14*	*51±3*	*60±5*	*59±5*	*70, 52*	*147*
Δ^8-cholestenol	*25±4*	*18±2*	*26±1*	*30±4*	*47, 39*	*80*
desmosterol	*160±10**	*84±4*	*211±9*	*141±14*	*329, 280*	*322*
lathosterol	*276±25**	*165±10*	*181±11*	*138±15*	*406, 417*	*418*
campesterol	*1051±114**	*542±29*	*1112±73*	*1049±94*	*935, 992*	*4970*
sitosterol	*470±47**	*266±15*	*489±140*	*542±52*	*398, 390*	*3387*
avenasterol	*135±10*[c]***	*91±7*[c]	*127±6*	*123±10*	*110, 129*	*487*
cholestanol	*418±21**	*228±8*	*460±22*	*408±33*	*515, 497*	*1059*
campestanol	*n.a.*	*n.a.*	*4±1*	*15±1*	*5, 0*	*19*
sitostanol	*n.a.*	*n.a.*	*6±1*	*18±2*	*15, 4*	*32*

Mean± SEM, except in Study IV; FH, familial hypercholesterolemia; HeZ, heterozygous FH; HoZ, homozygous FH; Hyperchol., hypercholesterolemic; n.a., not available; [a] *subjects from study V included,* [b] *mean of two measurements/person,* [c] *including also sitostanol, * $p<0.05$ vs. non-FH children*

Despite the marked differences in serum sterol concentrations, the respective ratios of non-cholesterol sterols to cholesterol in serum were similar in FH and unaffected children (Table 5). The serum ratios of cholesterol precursors to cholesterol were lower in statin-treated FH subjects than in the two subjects without statin treatment, whereas the ratios of serum precursor sterols of the homozygous subject were within the parents' upper limits. The serum ratios of plant sterols were higher in statin-treated FH compared with FH subjects without statin treatment. The ratio of cholestanol to cholesterol in serum was higher in subjects with the higher statin dose (atorvastatin $\geq$ 40 mg) than in the subjects with the lower dose (Figure 2, in Study III). In the subject with homozygous FH, the serum ratios of absorption marker sterols were as much as 10 times greater (twice for cholestanol) compared with the heterozygous parents, and two to five times higher than in the statin-treated FH subjects.

In the combined group of FH and non-FH children of Study I, the serum ratio of sitosterol to cholesterol was lower with apoE-4 phenotype than in those with other phenotypes (151±9 vs. 186±11 10^2 x mmol/mol of cholesterol). The squalene and non-cholesterol sterol levels in serum were similar between the genders and subjects with different LDL apoB receptor mutations in all studies. In children of Study I, the ratios of the precursor sterols in serum (e.g., lathosterol vs. Δ^8-cholestenol $r = 0.664$, $p<0.01$ in the FH group and $r = 0.743$, $p<0.001$ in the non-FH group) and also those of absorption markers in serum (e.g., cholestanol vs. sitosterol, $r = 0.532$, $p<0.05$ and r =

0.647, p<0.001, respectively) were correlated with each other. In children of Study II, only absorption markers in plasma were correlated with each other (e.g., campesterol vs. cholestanol r = 0.581, p<0.05). In statin-treated FH subjects (Study III), positive correlations were seen within serum cholesterol precursor sterols (e.g., lathosterol vs. Δ^8-cholestenol r = 0.753, p<0.001) and within absorption marker sterols in serum (e.g., campesterol vs. sitosterol r = 0.947, p<0.001). The negative correlation between plasma cholesterol precursors and absorption marker sterols was obtained only in Study II (e.g., campesterol vs. Δ^8-cholestenol r = -0.471, p<0.05).

Table 5. Baseline ratios of squalene and non-cholesterol sterols to cholesterol (10^2 x mmol/mol of cholesterol)

Variable	***Study I***		***Study II***	***Study III***[a]	***Study IV***	
	FH	*non-FH*	*Hyperchol.*	*FH with statin*	*Family Study*	
	Children		*Children*	*Adults*	*HeZ*	*HoZ*
	n=18	*n=29*	*n=23*	*n=18*	*n=2*[b]	*n=1*
squalene	*24±5**	*34±2*	*25±2*	*28±2*	*18, 15*	*44*
Δ^8-cholestenol	*9±2*	*12±1*	*11±0.5*	*14±2*	*13, 11*	*24*
desmosterol	*60±3*	*55±2*	*85±2*	*67±7*	*87, 81*	*97*
lathosterol	*102±7*	*108±6*	*73±4*	*66±7*	*110, 120*	*125*
campesterol	*393±36*	*358±19*	*451±29*	*508±50*	*250, 287*	*1491*
sitosterol	*176±14*	*177±11*	*198±11*	*265±29*	*106, 113*	*1016*
avenasterol	*51±3*[c]	*60±4*[c]	*51±2*	*60±6*	*29, 37*	*146*
cholestanol	*157±5*	*151±5*	*186±6*	*195±33*	*136, 144*	*318*
campestanol	*n.a.*	*n.a.*	*2±0.3*	*7±1*	*1, 0*	*5*
sitostanol	*n.a.*	*n.a.*	*2±1*	*9±1*	*4, 1*	*10*

Mean± SEM, except in Study IV; FH, familial hypercholesterolemia; HeZ, heterozygous FH; HoZ, homozygous FH; Hyperchol., hypercholesterolemic; n.a., not available; [a] *subjects from study V included,* [b] *mean of two measurements,* [c] *including also sitostanol, * p<0.05 vs. non-FH children*

To conclude, serum concentrations of non-cholesterol sterols were higher in FH children than in their normolipidemic controls, but when related to cholesterol, there was no difference between the FH and control children. The serum plant sterol and cholestanol levels were higher in subjects on statin treatment. The serum plant sterol levels were up to six times higher in the homozygous FH subject than in statin-treated heterozygous FH subjects. The positive correlations within cholesterol precursor sterols as well as absorption marker sterols suggest that these markers serve as indicators of cholesterol synthesis and absorption respectively also in this study population.

5.2.3. Lipoprotein non-cholesterol sterols

In absolute concentrations, LDL and HDL transported most of squalene and non-cholesterol sterols both in children and adults with FH and in healthy children (Table 3, in Study I). In the heterozygous FH children, ~75% of the non-cholesterol sterols were carried by LDL, ~20% by HDL, and <10% by VLDL and IDL. In non-FH children, the respective proportions were ~55%, 35-40%, and <10% (Figure 4). In statin-treated FH subjects, 62-75% of the non-cholesterol sterols were carried by LDL, whereas in the homozygous subject, up to 92% of non-cholesterol sterols were transported by the extensively large LDL. 49%, 41%, 45%, and 70% of squalene were transported by LDL in FH children, in non-affected children, in statin-treated FH adults, and in homozygous FH subject, respectively, whereas up to 27% of squalene was transported by TRL.

As ratios to cholesterol, the highest values of squalene and lathosterol, were obtained in VLDL and IDL in FH and non-FH children (Table 4, in Study I) and in VLDL and CM in adults with FH (Table 4, in Study III and Figure 3, in Study IV) and in the homozygous FH subject (Table 2 and Figure 3, in Study IV). The values of the precursor sterols in VLDL were mostly higher than the respective values in serum. The absorption marker sterols, sitosterol and cholestanol, less so campesterol, were most abundant in HDL and significantly higher than the respective ratios in serum (Table 4, in Study I; Table 4, in Study III; Table 2 and Figure 2, in Study IV). The lowest levels of absorption marker sterols were obtained in VLDL, IDL, and CM, and the values were significantly lower than the respective ratios in serum.

Thus, the concentrations of most of the non-cholesterol sterols were higher in FH, and LDL transported the highest proportion of non-cholesterol sterols. This suggests that LDL predicts, in addition to cholesterol, also the serum concentrations of non-cholesterol sterols. As with the ratios to cholesterol, the non-cholesterol sterol values were similar in FH and non-FH children. Cholesterol precursors were highest in VLDL, whereas cholesterol absorption marker sterols were most abundant in HDL.

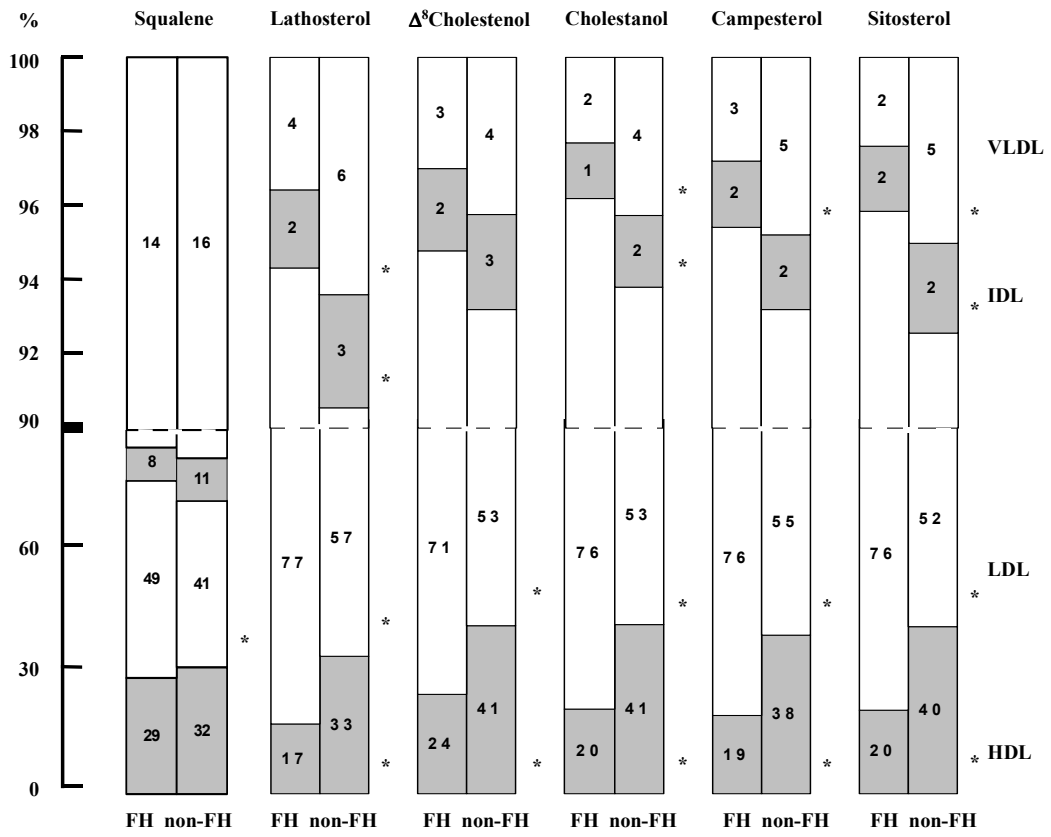

Figure 4. Percentage distribution of absolute amounts of squalene and non-cholesterol sterols in lipoproteins of FH (n=18) and non-FH (n=29) children (Study I).
*For squalene, n=14 and n=24, respectively. Note that ordinate scale is changed at 90. * p<0.05 FH vs. non-FH.*

5.2.4. Red cell sterols

Baseline ratios of squalene and non-cholesterol sterols to cholesterol in red cells of the hypercholesterolemic children (Study II) and statin-treated FH (Study III) are shown in Table 6.

In general, the baseline values of all non-cholesterol sterols in red cells, but not that of cholesterol, were correlated with the respective values in plasma or serum (Table 6).

Table 6. Baseline cholesterol (mg/dl), squalene, and non-cholesterol sterol ratios to cholesterol in red cells (10^2 x mmol/mol of cholesterol) and their correlation coefficients (r) to respective values in plasma (Study II) or in serum (Study III).

Variable	***Study II*** *Hyperchol. Children n=23*	***r***	***Study III***[a] *FH + statin Adults n=18*	***r***
cholesterol	*114±2*	*-0.212*	*108±2*	*0.241*
squalene	*34±6*	*0.131*	*41±4*	*-0.198*
Δ^8-cholestenol	*9±1*	*-0.492**	*4±2*	*0.480**
desmosterol	*59±2*	*0.497**	*63±10*	*0.740**
lathosterol	*199±10*	*0.767**	*132±17*	*0.961**
campesterol	*526±30*	*0.896**	*580±58*	*0.995**
sitosterol	*231±63*	*0.923**	*304±33*	*0.995**
avenasterol	*62±3*	*0.455**	*74±6*	*0.969**
cholestanol	*237±12*	*0.508**	*190±14*	*0.890**
campestanol	*0.4±0.2*	*-0.195*	*4±1*	*0.428*
sitostanol	*8±2*	*-0.332*	*9±1*	*0.090*

*Mean±SEM; FH, familial hypercholesterolemia; Hyperchol., hypercholesterolemic; [a]subjects from study V are included, * $p<0.05$*

5.3. Effects of phytosterols

Dietary phytosterols lower serum cholesterol, and affect the serum non-cholesterol sterol values. However, the effects of especially plant sterol esters on serum non-cholesterol sterols in hypercholesterolemic children, homozygous FH, and statin-treated FH subjects have not been evaluated in detail. In addition, the data on the effects of phytosterol esters on red cell and lipoprotein non-cholesterol sterols are limited. Therefore, we measured serum, lipoprotein, and red cell non-cholesterol sterols during consumption of phytosterol esters in the present study populations. We were especially interested in changes occurring in plant sterol levels.

5.3.1. Serum and lipoprotein lipids

The effects of plant stanol and sterol ester spreads on serum, LDL, and HDL cholesterol and serum TG levels in hypercholesterolemic children (Study II) and in statin-treated FH (Study III) are shown in Figure 5. Both spreads lowered total and LDL cholesterol by 6-15%, but only the plant sterol ester spread significantly increased HDL cholesterol and decreased TG in statin-treated FH subjects in the present study.

In children, the ingested amounts of plant stanol and sterol ester spreads correlated positively ($r = 0.448$ and $r = 0.428$, respectively, $p<0.05$ for both) with the decrease in LDL cholesterol. In statin-treated FH, the baseline LDL cholesterol was negatively correlated with its respective change i.e., the higher the baseline LDL cholesterol levels, the larger its reduction evoked by both spreads (Figure 6). Only in children, did the baseline campesterol ratio in plasma predict the subsequent decrease in the concentrations of total and LDL cholesterol during consumption of the plant sterol ester spread ($r = 0.421$ and $r = 0.452$, respectively, $p<0.05$ for both).

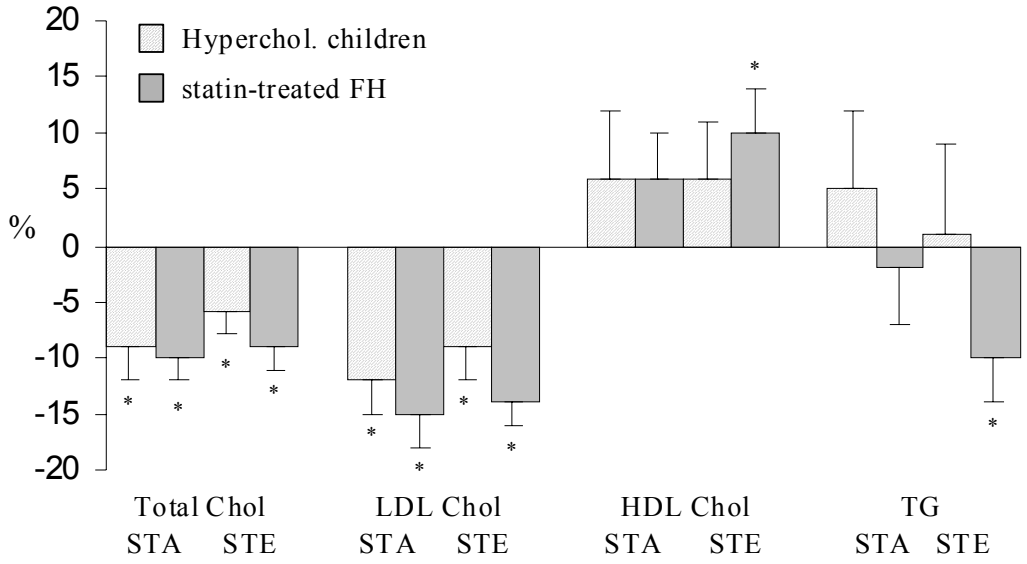

Figure 5. Percentual changes of serum lipids in hypercholesterolemic children (Study II, n=23) and in statin-treated FH subjects (Study III, n=18) during consumption of plant stanol (STA) and sterol (STE) ester spreads.
** p<0.05 vs. baseline. Chol, cholesterol.*

In the homozygous FH subject (Study IV), the longer the interval between apheresis, the greater the pre-apheresis concentrations of serum total and LDL cholesterol (r = 0.885 and 0.913, respectively, p<0.001). Comparison of the lipid values at similar apheresis intervals revealed ca. 8% and 9% reduction in total and LDL cholesterol, respectively by both spreads (Table 1, in Study IV). In the heterozygous parents, both spreads decreased on average 14% and 17% of the total and LDL cholesterol (Table 1, in Study IV).

These results suggest that plant stanol and sterol ester spreads are equally effective in lowering LDL cholesterol in hypercholesterolemic children and in statin-treated FH subjects in short-term studies. Further, the results indicate that in the statin-treated FH subjects the greatest decrease in LDL cholesterol was obtained in those subjects with the highest baseline levels of LDL cholesterol, whereas in hypercholesterolemic children the baseline plasma campesterol ratio predicted the best response to plant sterol ester spread.

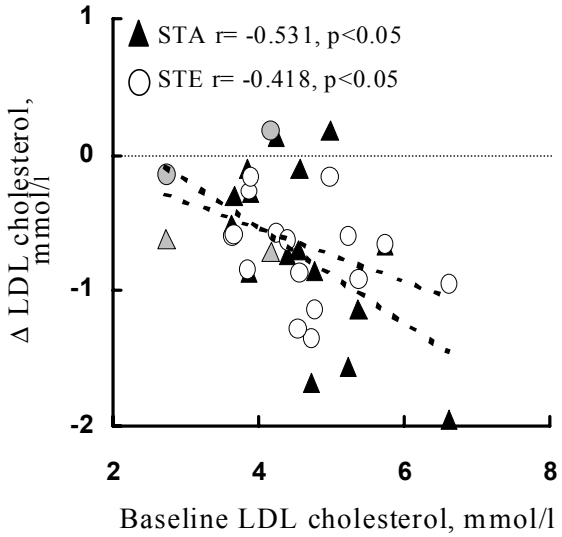

Figure 6. Correlation between baseline LDL cholesterol to respective change in LDL cholesterol during consumption of plant stanol (STA) and sterol (STE) ester spreads in statin-treated FH subjects (Study III, n=18).
The gray-colored symbols depict subjects having also bile acid sequestrants.

5.3.2. Serum and lipoprotein non-cholesterol sterols

Cholesterol precursors

Consumption of both spreads increased serum ratios of cholesterol precursor sterols, significantly so for desmosterol and lathosterol, in hypercholesterolemic children (Study II), in statin-treated FH subjects (Study III) (Figure 7), as well as in two FH subjects without statin treatment (Figure 3, in Study IV). The lower the baseline ratios of the precursors of cholesterol, the higher were their increments with both spreads in hypercholesterolemic children (r ranged from –0.229 to -0.617) and statin-treated FH subjects (r ranged from –0.457 to –0.820, except lathosterol). In the homozygous subject, the precursor sterol ratios appeared to be decreased by the sterol ester spread but increased in VLDL by the stanol ester spread (Figure 3, in Study IV).

No changes occurred in cholesterol precursor concentrations or ratios to cholesterol in different lipoproteins during the interventions in statin-treated FH subjects (Table 4, in Study III).

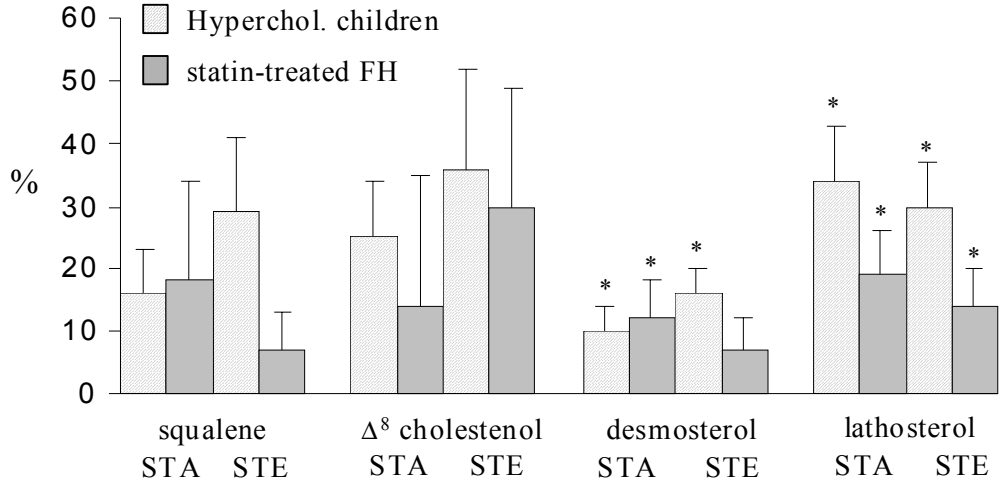

Figure 7. Percentual changes of squalene and cholesterol precursor sterols in hypercholesterolemic children (Study II, n=23) and in statin-treated FH subjects (Study III, n=18) during consumption of plant stanol (STA) and sterol (STE) ester spreads.
** p<0.05 vs. baseline.*

Phytosterols and cholestanol

Consumption of the plant sterol ester spread increased serum campesterol and sitosterol ratios in hypercholesterolemic children and in statin-treated FH subjects, whereas the respective values were decreased by the plant stanol ester spread (Figure 8). Serum avenasterol levels were decreased in children by both spreads, but only by plant stanol ester spread in FH adults. In absolute concentrations, plant sterol ester spread increased the total amount of plant sterols from 1.5 mg/dl up to 2.3 mg/dl in children (Study II) and from 1.5 mg/dl to 2.4 mg/dl in statin-treated FH subjects. The plant stanol ester spread increased serum plant stanol concentrations several times, but the values remained small (e.g., campestanol 19±2 μg/dl in children and 35±3 μg/dl in statin-treated FH). Both spreads decreased serum cholestanol levels, but this was significant only for the plant sterol ester spread.

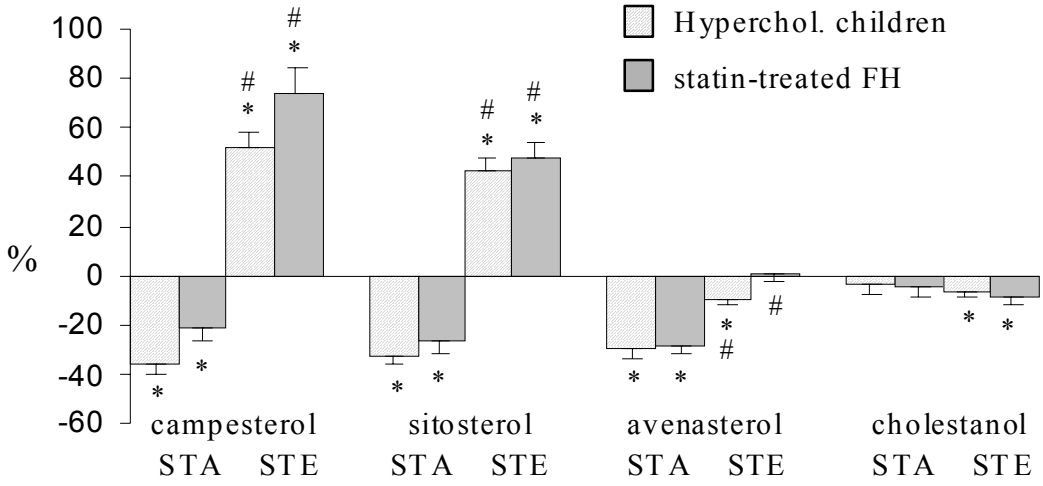

Figure 8. Percentual changes of plant sterols and cholestanol in hypercholesterolemic children (Study II, n=23) and in statin-treated FH subjects (Study III, n=18) during consumption of plant stanol (STA) and sterol (STE) ester spreads.
**p<0.05 vs. baseline, # p<0.001 STE vs. STA*

The baseline ratios of plasma campesterol and sitosterol predicted their respective changes during consumption of the spreads, such that the plant sterol ratios decreased most in subjects with the highest respective baseline levels during plant stanol ester spread in children (Study II) (e.g., r = -0.625 for campesterol, $p<0.05$) and in statin-treated FH subjects (Study III) (Figure 9 for sitosterol). In statin-treated FH subjects, during consumption of the sterol ester spread, the increase of sitosterol (Figure 9) and campesterol was greatest in subjects with the highest baseline levels. In addition, a similar association was observed in the children of Study II (e.g., r = 0.489 for sitosterol, $p<0.05$). A more detailed analysis revealed that the increase of serum plant sterols was greatest in adult FH subjects with the highest statin doses. The change of serum cholestanol was negatively correlated with its baseline levels with both spreads in both study groups (r ranged from –0.361 to –0.632) (Figure 1, in Study III).

In statin-treated FH subjects, the concentrations of campesterol and sitosterol were increased in CM, LDL, and HDL during consumption of plant sterol ester spread (Table 4, in Study III). However, the percentual proportion of campesterol and sitosterol was decreased in LDL and increased in HDL by both spreads (Figure 10a for campesterol). When the results were calculated as ratios to cholesterol, the percentual increments of plant sterols by plant sterol ester consumption, and the respective decrements by plant stanol consumption were similar in all lipoproteins (Figure 10b; Table 4, in Study III).

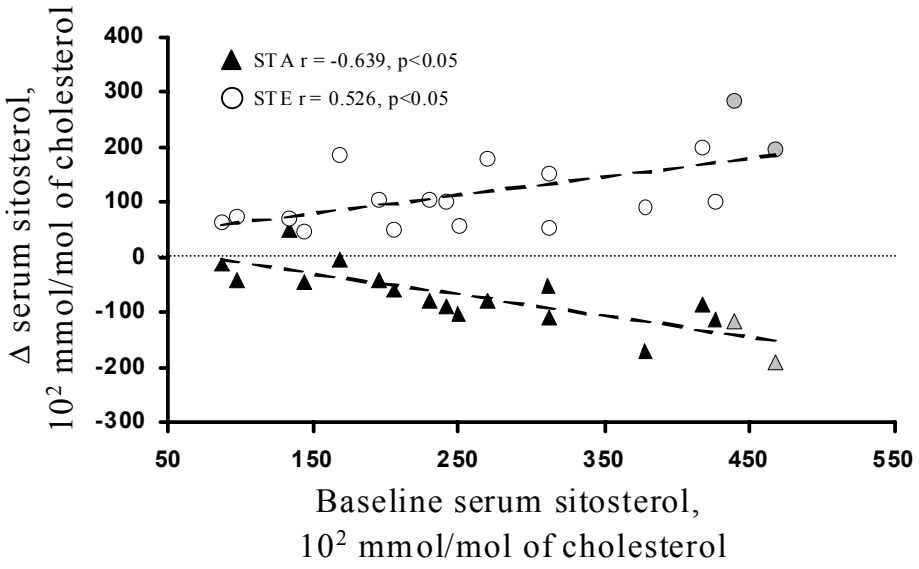

Figure 9. Correlations between baseline serum sitosterol to cholesterol ratio and change in serum sitosterol ratio to cholesterol during consumption of plant stanol (STA) and sterol (STE) ester spreads in statin-treated FH subjects (Study III). *The gray-colored marks depict subjects having also bile acid sequestrants.*

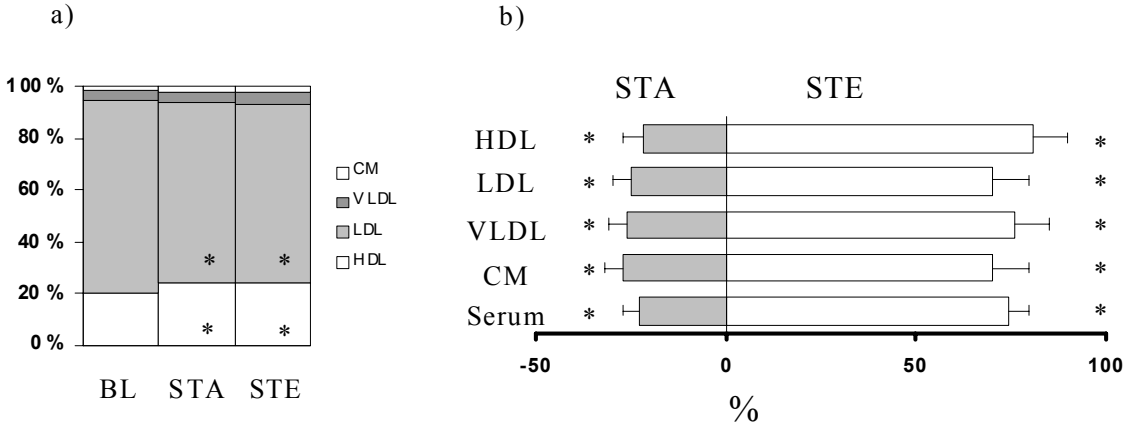

Figure 10. Campesterol in different lipoproteins.

a) The percentage distribution of absolute amounts of campesterol in different lipoproteins of statin-treated FH subjects at baseline (BL) and during consumption of plant stanol (STA) and sterol (STE) ester spreads. * *p<0.05 vs. baseline.*

b) The percentual changes of campesterol ratios to cholesterol in serum and different lipoproteins during consumption of plant stanol (STA) and sterol (STE) ester spreads. * *p<0.05 vs. baseline.*

In the homozygous FH subject, plant sterol ester spread increased the serum ratios of plant sterols by ~30%, and the changes were similar in all lipoproteins (Figure 11 for sitosterol). The concentration of serum total plant sterols was increased up to 14 mg/dl, and the increase occurred mainly in LDL (Figure 11 for campesterol). During consumption of plant stanol ester spread, the levels of plant sterols were decreased even below the values obtained during consumption of the home diet. The concentration of cholestanol and its ratio to cholesterol were reduced by both spreads by ~15%. Plant stanol concentrations were increased up to 0.3 mg/dl in serum by plant stanol ester spread.

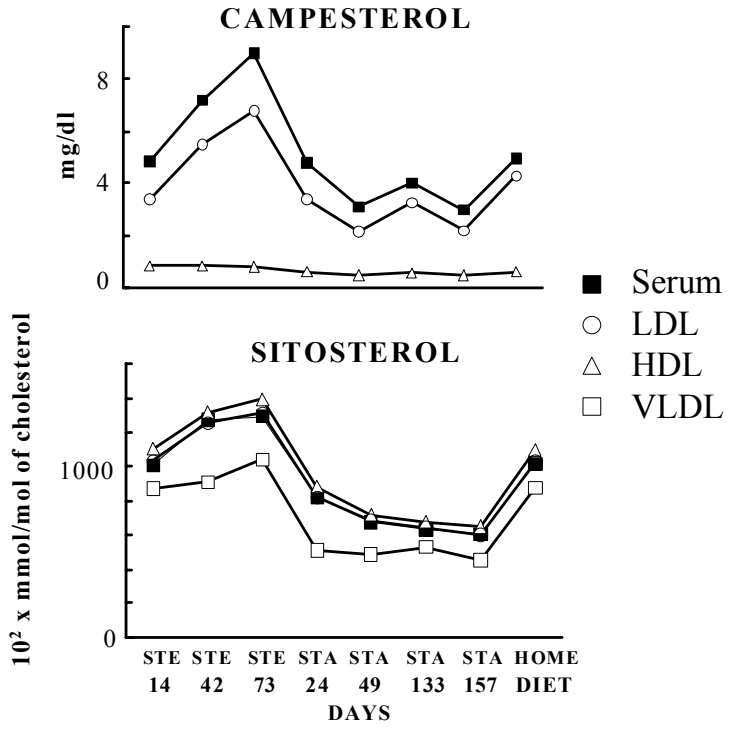

Figure 11. Concentration of campesterol and ratio of sitosterol to cholesterol in serum and different lipoproteins of the homozygous FH subject on home diet and during consumption of plant sterol (STE) and stanol (STA) ester spreads (Study IV).

To conclude, dietary plant sterol ester spread increased the serum and lipoprotein plant sterol levels in all study populations, whereas the dietary plant stanol ester spread decreased the respective values. The changes occurred similarly in all lipoproteins. The baseline serum levels of plant sterols predicted the respective change during consumption of phytosterol esters, such that the serum plant sterol levels were increased most in those subjects with the highest serum baseline plant sterol levels, whereas consumption of plant stanol ester spread resulted in the greatest

decrease of serum plant sterols in the same subjects. The changes in serum plant sterols did not correlate with the changes in LDL cholesterol.

5.3.3. Red cell sterols

The red cell cholesterol concentrations remained constant during the intervention periods both in hypercholesterolemic children and in statin-treated FH subjects. Neither the total nor the free serum cholesterol could be correlated with the red cell cholesterol. The changes in the ratios of cholesterol precursor sterols to cholesterol in red cells were inconsistent in the two study populations; the ratio of lathosterol to cholesterol was increased by ~20% in both studies, whereas the desmosterol ratio was increased by ~15% only in Study II. Negative correlations were obtained between most baseline cholesterol precursors and their respective change with both spreads in both study populations (e.g., r ranged from –0.747 to –0.813 for red cell Δ^8-cholestenol). The changes of cholesterol precursors in red cells were mostly correlated with the changes in serum (Table V, in Study II; Figure 12b for lathosterol of the combined study population).

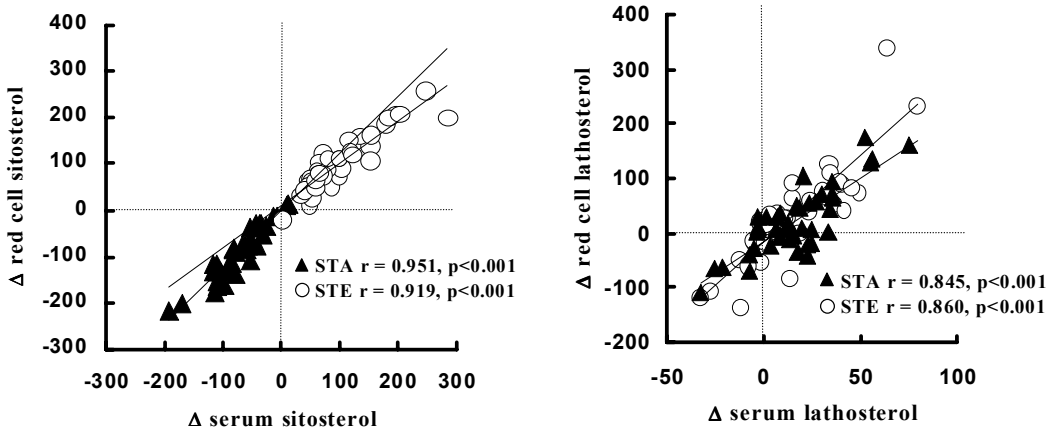

Figure 12. Correlations between changes of serum and red cell campesterol (a) and lathosterol (b) to cholesterol ratios (10^2 mmol/mol of cholesterol) during consumption of plant stanol (STA) and sterol (STE) ester spreads of combined study population (hypercholesterolemic children, Study II, n=23 and statin-treated FH, Study III, n=18). For sitosterol, y=0.9x+8.7 (STE), y=1.2x-1 (STA), and for lathosterol, y=3.1x-15 (STE), y=2.3x-14 (STA).

The ratios of campesterol and sitosterol to cholesterol were decreased in red cells by ~20-35% during consumption of plant stanol esters, and increased by ~40-75% during consumption of plant sterol esters (Table 4, in Study II and Table 3, in Study III). The changes of the campesterol and sitosterol ratios to cholesterol in red cells correlated with the respective changes in plasma or serum (Figure 12a for sitosterol in the combined study population). The baseline plant sterol levels in red cells were negatively correlated with the change of plant sterols in red cells in both study populations during consumption of the stanol ester spread (e.g., r ranged from –0.652 to –0.691 for campesterol; Table 3, in Study II). Only insignificant positive correlations between baseline red cell plant sterol levels and the dietary plant sterol

ester-induced increase were observed in both study populations (e.g., r ranged from 0.360 to 0.371 for sitosterol). Red cell content of plant stanols was increased by the stanol ester spread, but the actual amounts remained small (e.g., 25 μg/dl and 41 μg/dl for sitostanol in hypercholesterolemic children and statin-treated FH subjects, respectively).

Thus, the changes of plant sterols in red cells correlated highly with the respective change in serum or in plasma occurring during phytosterol ester treatment in both study populations.

5.3.4. Removal of intravenous Intralipid

In FH, the clearance of postprandial lipoproteins may be impaired in general, but it is improved by statin therapy. In order to determine, whether inhibition of cholesterol absorption by dietary phytosterols further could alter the clearance of postprandial lipoproteins, we studied the removal of intravenous Intralipid+squalene emulsion during consumption of dietary phytosterol esters.

Serum total and LDL cholesterol concentrations were significantly decreased ($p<0.05$-0.01) by both phytosterol-enriched spreads in the five subjects of this substudy, whereas HDL cholesterol and TG remained stable (Table 1, in Study V). CM and VLDL cholesterol remained unchanged between the periods, while CM TG was increased by both spreads. The sterol ester spread increased and the stanol ester spread decreased the concentrations of plant sterols in serum and CM. No significant changes were seen in serum, CM, and VLDL squalene levels between the dietary intervention periods (Table 2, in Study V).

The Intralipid-squalene fat load test was performed at baseline during statin therapy and during consumption of plant stanol and sterol ester spreads. The incremental concentrations of cholesterol, TG, squalene, and sitosterol were subtracted from the baseline levels. These incremental curves are shown in Figure 13.

At baseline and during the intervention periods, the peak concentrations of cholesterol, TG, squalene, and plant sterols in CM were reached at 10 min. In VLDL, the peak level of sitosterol and squalene was reached at 10 to 20 min, and of TG at 60 min, while no significant increments of cholesterol and campesterol occurred in VLDL. The incremental curves for CM and VLDL cholesterol and TG were rather similar during consumption of the stanol and sterol ester spreads than at baseline (Figure 13). The peak concentration of sitosterol at 10 min was higher during consumption of the spreads than at baseline, and the increments of both campesterol and sitosterol at 120 min were higher during the stanol ester spread than at baseline. The AUICs of cholesterol, TG, and plant sterols in CM and VLDL were similar at baseline and during the intervention periods.

The incremental concentrations of CM squalene were higher up to 120-180 min during the two spread periods vs. baseline, and at 180 min the incremental concentrations still exceeded the starting values (Figure 13). The AUICs of CM squalene were similarly higher with both spreads than at baseline (4252±917 at

baseline, 7424±818 by stanol esters, and 7810±688 by sterol esters, $p<0.05$ by ANOVA). The higher the incremental squalene concentration at 10 min, the higher the AUIC of the combined (baseline+stanol+sterol) values ($r=0.836$, $p<0.05$). The incremental concentrations of VLDL squalene were higher in some measurement points only for the stanol ester spread vs. baseline (Figure 13). The AUICs of VLDL squalene were only insignificantly higher with both spreads compared with baseline.

Clearance of CM squalene and campesterol was slower than that of sitosterol and TG during all periods (Figure 6, in Study V). The clearance curves of squalene, plant sterols, and TG were similar between the periods, and the half-lives of different compounds did not differ between the periods. The baseline sitosterol (0.748, $p<0.01$) was positively correlated with the respective half-lives.

Thus, removal of squalene and less so plant sterols was unexpectedly impaired during consumption of phytosterol esters in statin-treated FH subjects. However, the clearance curves and half-lives of the different compounds did not differ between the three periods. The altered plant sterol concentrations in serum by the spreads did not affect the removal of plant sterols from CM or VLDL.

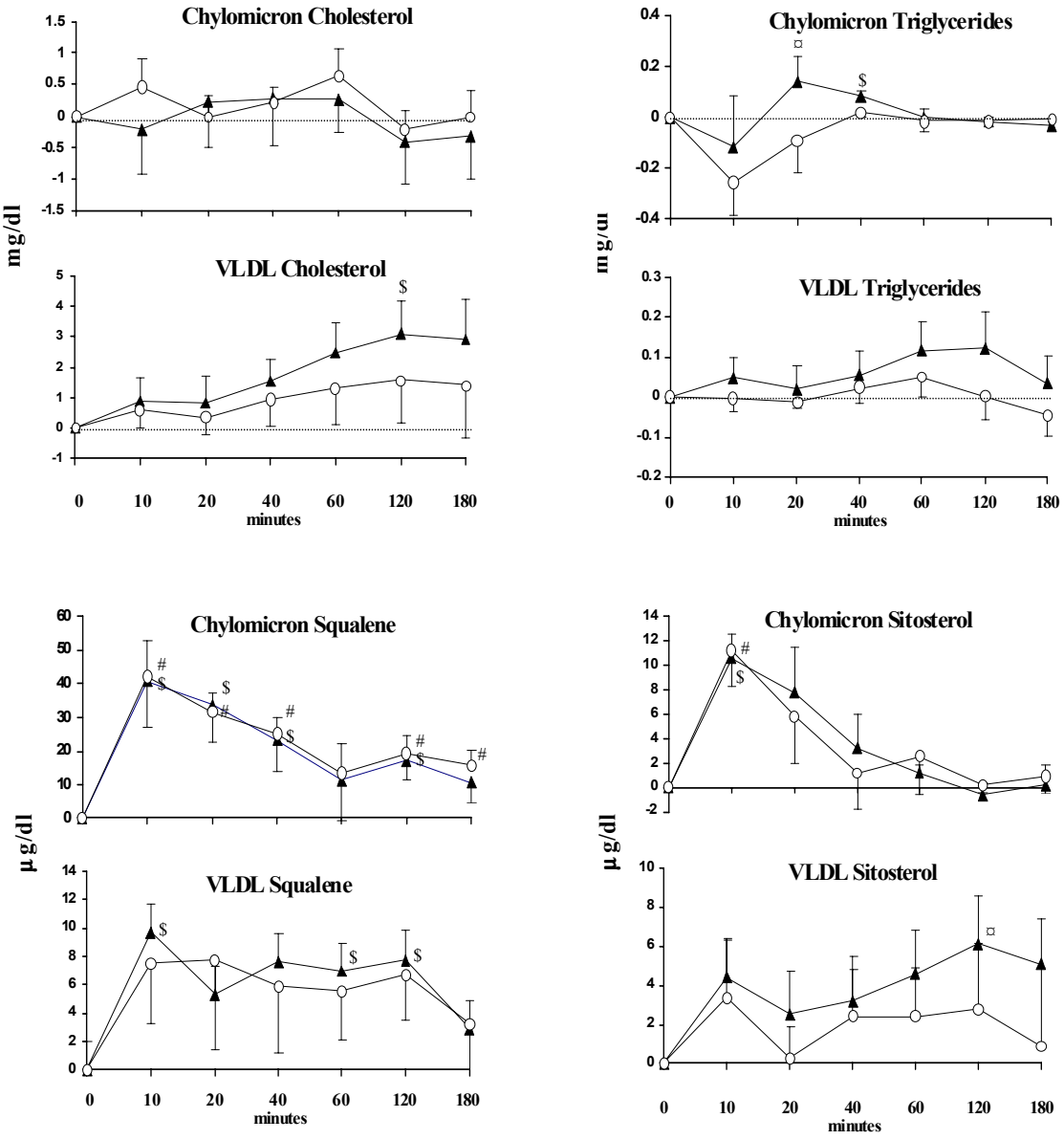

Figure 13. The differences between incremental concentrations during consumption of plant stanol (closed triangle) and sterol (open circle) ester spreads vs. baseline (level 0) for chylomicron and VLDL cholesterol, triglycerides, squalene, and sitosterol in statin-treated FH subjects.
Results represent means±SEM. Incremental concentration different from baseline, $ $p<0.05$ by stanol esters, # $p<0.05$ by sterol esters, and ¤ $p<0.05$ from sterol esters by stanol esters.

6. DISCUSSION

6.1. Study population and design

Altogether 88 subjects, including 39 males and 49 females with an age-range of 1 to 62 years, were recruited into the present study. Fifty-one of the subjects had DNA diagnosed FH, and the clinical picture and family history of a further two subjects (one child and one adult) were appropriate to FH, and thus they were included in the FH group. Six children without FH in Study II were hypercholesterolemic with similar serum cholesterol levels as the FH children, and were thus included in the intervention study. Three children from Study I participated also in Study II, and five adult subjects of Study V also took part of Study III. Only prepubertic children were included in Study II, since during pubertal development, some changes in serum lipids may occur (Porkka et al. 1994). The negative DNA diagnosis of the 29 normolipidemic subjects in Study I suggested that they were non-affected members of FH families. However, the present normolipidemic subjects cannot be considered as a normal control population, since dietary recommendations given for all the FH families could have affected the dietary intake of fat and cholesterol and subsequently lowered the serum lipid levels of non-affected children as well.

Study III consisted of FH subjects on statin therapy. For ethical reasons we decided not to study adults with FH without statin treatment. The two subjects in Study IV had refused to start statin therapy but were amenable to dietary treatment. In Study III, the prior statin treatment was kept unchanged. All subjects had used their statin treatment for many years, such that the steady state of cholesterol metabolism had been reached. Since the subjects were their own controls, the possible effects of confounding factors, e.g., different statin preparations and doses and possible additional treatment, such as bile acid sequestrants or hormone replacement therapy were thus kept to a minimum. Study IV was a family study including only three subjects. Due to the low prevalence of homozygous FH, a study with a larger number of homozygous FH adults is impossible to do in Finland. Accordingly, no statistical calculations could be performed.

In Study I, the subjects visited the Outpatient Clinic once and only one blood sample was obtained. Due to the young age of some study subjects, the numbers of visits and blood samples were kept to a minimum. Studies II-V had a cross over design. Study II had a wash-out period, but Studies III-V were conducted without any wash-out period. The lack of wash-out period in Studies III-V was taken into account in the statistical calculations by evaluating the effect of treatment period and sequence of treatment.

In the homozygous FH subject, LDL apheresis was scheduled to occur at fortnightly intervals, but for the patient-related reasons, the periods were sometimes prolonged. Since apheresis is followed by increased levels of serum sterols, including plant sterols, the prolonged intervals inevitably affected the results. However, we analyzed the data also by adjusting serum non-cholesterol sterols to the respective cholesterol levels. Since we could not make any statistical calculations between the homo- and

heterozygotes, this study gives only a profile of one homozygous subject compared with the heterozygous parents.

The compliance in the intervention studies was good. In addition, increased serum plant stanol and sterol levels during the interventions, as well as increased serum cholesterol precursor sterols served as markers for good compliance.

6.2. Serum and lipoprotein lipids

6.2.1. Baseline

The impaired LDL apoB receptor in FH is accompanied with high serum total and LDL cholesterol levels (Goldstein et al. 2001), also in children (Kwiterovich 1989). Thus, as expected, serum total and LDL cholesterol levels were higher in the FH than in the non-FH children (Study I). The serum lipid values of the two groups were definitely different, since there was no overlapping in the lipid values; the lowest LDL cholesterol value in FH was 3.89 mmol/l, whereas the highest LDL cholesterol value in non-affected children of same age was 2.92 mmol/l.

ApoE phenotype affects the serum total and LDL cholesterol levels in non-FH adults (Davignon et al. 1988) and in normocholesterolemic children (Kallio et al. 1997). However, no association between serum total and LDL cholesterol and apoE phenotype has been obtained in adults (Gylling et al. 1989a) or children (Wiegman et al. 2003) with FH. We obtained similar findings in the present study. In the children without FH, the highest serum total and LDL cholesterol values were obtained in the children with apoE-4 phenotype, whereas no association between serum total and LDL cholesterol and apoE phenotype was present in children with FH. The authors from a previous study have speculated that the presence of the mutation in LDL apoB receptor gene could overrule other factors, including apoE phenotype (Wiegman et al. 2003).

The majority of the patients on statin therapy do not achieve the recommended LDL cholesterol levels (Andrews et al. 2001). Thus, it was not surprising that despite ongoing statin therapy in Study III, the serum total and LDL cholesterol values were above the recommended levels (5.0 mmol/l and 3.0 mmol/l, respectively) according to European guidelines (De Backer et al. 2003). Taking into account the fact that all the subjects had FH, the statin doses used by the present study subjects were surprisingly low. Accordingly, especially in FH, higher statin doses should be used to achieve the recommended serum lipid values. In fact, when the present statin-treated FH subjects were divided into high- and low-statin groups, LDL cholesterol levels were as much as 1 mmol/l lower in the high-statin group compared with subjects with lower statin dose. The difficulties to achieve tolerable LDL cholesterol values in homozygous FH subjects could also be seen in the present study. Thus, despite aggressive lipid-lowering therapy given to the present homozygous FH subject, the total and LDL cholesterol levels were at the same level as in the heterozygous parents who were not taking any hypolipidemic medication (Study IV).

6.2.2. Effects of phytosterols

The cholesterol lowering effect of dietary phytosterols on serum lipid levels has been demonstrated in several earlier studies reviewed recently by Katan et al. (2003). These effects have mostly been studied by short-term interventions, as also in the present study. However, only two long-term studies, one with plant stanol esters (Miettinen et al. 1995) and the other one with plant sterol esters (Hendriks et al. 2003) have been conducted. The consumption of plant stanol esters for one year in hypercholesterolemic subjects led to ~9% and ~13% reduction in LDL cholesterol with doses of 1.8 g/day and 2.6 g/day of plant stanols (Miettinen et al. 1995), whereas consumption of lower dose of plant sterol esters (1.6 g/day of plant sterols) reduced LDL cholesterol by ~6% (Hendriks et al. 2003) when compared with placebo group. Closer inspection of the results showed that the LDL cholesterol reduction did not persist in the long-term use, and accordingly the mean LDL cholesterol value after one year's consumption of plant sterols was reduced (5%) from the baseline value only in men (Brink and Hendricks 2000). Accordingly, the short-term studies, including the present intervention study, demonstrate only the cholesterol lowering effect of phytosterol esters over short-term use. Thus, one cannot draw any conclusions on the long-term benefit of dietary phytosterol esters from short-term studies.

In children, dietary phytosterols offer a well-tolerated means to lower effectively serum total and LDL cholesterol. In particular, the treatment of children with FH should be started as early as possible, and dietary phytosterol esters are suitable and effective prior to statin therapy. The present 12% and 9% reductions obtained in LDL cholesterol by plant stanol and sterol esters were of the same magnitude as those obtained from previous studies in FH children (Gylling et al. 1995, Vuorio et al. 2000, Amundsen et al. 2002, de Jongh et al. 2003). Accordingly, it may be concluded from the present and earlier studies that plant stanol and sterol ester spreads are equally effective in hypercholesterolemic children in short-term studies, but their long-term efficacy has not been evaluated. In fact, in addition to long-term efficacy of phytosterol esters, the long-term effects of the possible reduction in the levels of fat soluble vitamins and especially antioxidants (Vuorio et al. 2000, Tammi et al. 2000) on growth of the children will need to be assessed.

Plant stanol esters have been shown to be most efficient in adult subjects with high baseline absorption of cholesterol (i.e., high baseline serum cholestanol ratio) (Gylling et al. 1997, Gylling and Miettinen 2002a). In the present study in children (Study II), high baseline cholesterol absorption predicted also efficient reduction of plasma total and LDL cholesterol, but this correlation was, however, seen only for the sterol ester spread. There were no correlations between cholesterol synthesis markers and reduction in plasma total or LDL cholesterol obtained by either spreads. This is in contrast with an earlier study with FH children consuming stanol esters (Gylling et al. 1995). In that study, the most efficient reduction of LDL cholesterol was demonstrated in those subjects with the highest baseline cholesterol synthesis. This discrepancy in results remains unclear, but may be related to the small number of study subjects. The study by Gylling et al. (1995) consisted of only subjects with FH,

whereas the present study group included also hypercholesterolemic children without FH. Thus, the effects of heterogeneous study population on the present results cannot be ruled out.

Adding dietary phytosterol esters to statin therapy offers an opportunity to affect both the synthesis of cholesterol and the intestinal absorption of dietary and biliary cholesterol. The additional reduction of 9-10% and 14-15% of serum total and LDL cholesterol with phytosterol esters in statin-treated FH subjects (Study III) is in accordance with previous short-term studies conducted with subjects on concomitant statin treatment (Table 1). Even though only one subject achieved the goal of LDL cholesterol <3.0 mmol/l, 2/3 of the subjects, instead of 1/3 prior to the dietary intervention, achieved LDL cholesterol level below 4.0 mmol/l. The present reduction in LDL cholesterol achieved by phytosterol esters was almost similar to the reductions obtained by ezetimibe (from 14% to 25%) when this drug has been added to ongoing statin therapy in mildly hypercholesterolemic subjects (Davidson et al. 2002, Gagné et al. 2002a). Ezetimibe also improved HDL cholesterol and TG values in statin-treated subjects. Even though some improvement in HDL cholesterol and TG concentrations has been obtained during consumption of phytosterol esters (Katan et al. 2003) as found also in the present study, phytosterols in general do not affect either HDL cholesterol or TG levels.

The negative correlation of baseline LDL cholesterol level with its respective change (Figure 6) suggests that the efficiency of phytosterol esters to lower LDL cholesterol is better in those subjects with higher baseline LDL cholesterol values. This would further suggest that subjects treated with a low dose of statin or those responding insufficiently to statin treatment e.g., due to high baseline cholesterol absorption efficiency (Miettinen et al. 2000a), may achieve optimal benefit from the cholesterol absorption inhibitor combined with statin.

The hypocholesterolemic effect of phytosterol esters was difficult to evaluate in the homozygous FH subject because of the varying LDL apheresis intervals. However, some reduction in LDL cholesterol (~9%) was detectable at the comparable two-week post-apheresis periods during stanol and sterol ester periods. Ezetimibe combined with statin decreased LDL cholesterol by about 20% in 33 homozygous FH patients, of whom some were on LDL apheresis treatment (Gagné et al. 2002b). These results suggest that an additional benefit can be obtained from the inhibition of cholesterol absorption even in individuals with homozygous FH.

6.3. Serum non-cholesterol sterols

We measured serum non-cholesterol sterols in FH and normocholesterolemic children in Study I, and compared the serum values with those in different lipoproteins. In addition, since earlier studies have demonstrated that dietary plant sterols in their free form can increase the serum plant sterols levels markedly especially in children (Mellies et al. 1976a), we evaluated the effects of dietary plant sterol esters on serum plant sterol levels in hypercholesterolemic children. In addition, the association of high serum plant sterol levels and CHD (Glueck et al. 1991, Sudhop et al. 2002a, Assmann et al. 2003) has raised the question of how high the serum plant sterol levels

may be increased at baseline and during consumption of phytosterol esters in special study populations, such as in homozygous FH and in heterozygous FH with statin treatment.

6.3.1. Baseline

The data from Study I showed that serum non-cholesterol sterol concentrations were markedly higher in FH than in normolipidemic children, similarly to the situation in FH adults (Gylling and Miettinen 1988). In addition, the highest concentrations of all non-cholesterol sterols were seen in the homozygous FH subject (Study IV). Thus, it seems that serum non-cholesterol sterol levels follow the respective cholesterol levels, and in addition to serum cholesterol levels, the grossly increased LDL fraction also determines the concentrations of non-cholesterol sterols.

Serum cholesterol precursors

Despite differences in concentrations of cholesterol precursor sterols, children with and without FH had similar serum ratios of cholesterol precursor sterols (Study I). Since serum levels of cholesterol precursor sterols correlate positively with cholesterol synthesis (Kempen et al. 1988, Miettinen et al. 1990b), the similar ratios of these precursor sterols in serum in FH and non-FH children suggest that cholesterol synthesis was similar in the two groups of children. In fact, similar findings have been obtained in an earlier study in children, in whom the cholesterol synthesis was evaluated by the sterol balance technique (Martin and Nestel 1979).

Surgical procedures performed in the homozygous FH subject (the portacaval shunt and ileal bypass operation) have apparently altered cholesterol synthesis (Miettinen 1979), but the effects of these procedures have possibly been modified by long-term statin and apheresis treatments, even though only slight and inconsistent changes in cholesterol synthesis have been reported during long-term apheresis treatment (Pfohl et al. 1994, Gylling et al. 1998). However, cholesterol precursor sterol ratios were similar in the homozygous offspring at baseline on current treatment and in the heterozygous parents without statin therapy. This would suggest that cholesterol synthesis was similar in the members of this FH family. Since statin treatment inhibits cholesterol synthesis and subsequently lowers cholesterol precursor sterol ratios in serum (Kempen et al. 1988, Uusitupa et al. 1992, Vanhanen and Miettinen 1992a, Vanhanen et al. 1992, Pfohl et al. 1998), the serum ratios of desmosterol and lathosterol were lower in statin-treated FH subjects than in the two FH subjects without statin treatment, and thus also lower than in the homozygous FH subject. Accordingly, it seems that despite high-dose statin treatment, cholesterol synthesis was upregulated in the homozygous subject, due to altered metabolic state caused by LDL apheresis treatment and surgical procedures etc.

Serum phytosterols and cholestanol

In healthy subjects, the total plasma plant sterols range from 0.3 to 1.0 mg/dl (Björkhem et al. 2001). Accordingly, the serum total plant sterol concentration of ~0.9 mg/dl of the non-FH children was within the normal limits. However, even at

baseline, the mean total serum plant sterol concentration up to ~1.5 mg/dl in all FH subjects was doubled compared with non-affected children, and in the homozygous subject the total serum plant sterol concentration (~9 mg/dl) was up to ten times higher than in normolipidemic children. Accordingly, FH children without any medication as well as FH adults with statin therapy and homozygous FH subject of the present study all had increased serum plant sterol levels at baseline. Whether this increased serum plant sterol level increases the risk for CHD in these subjects, is unknown.

In adults with FH, the serum plant sterol and cholestanol ratios to cholesterol have been shown to be higher than in a randomly selected Finnish male population (Gylling and Miettinen 1988). In contrast, Vuorio et al. (2002) demonstrated that at the age of one year, all the serum non-cholesterol sterols were similar in FH and non-FH-children. Similarly, the present study showed that ratios of serum plant sterols and cholestanol to cholesterol were similar in children with FH compared to their non-affected controls. Cholesterol absorption efficiency has earlier been studied for ethical reasons only in adult subjects with FH. These studies have demonstrated that the absorption of cholesterol was similar in subjects with FH and their controls (Connor and Lin 1974, Gylling and Miettinen 1989). Accordingly, similar to the situation in adults with FH, the present data suggest that there is no difference in the cholesterol absorption efficiency between the children with FH and their normolipidemic controls.

ApoE phenotype has been postulated to control cholesterol absorption efficiency in Finnish modestly hypercholesterolemic adults (Kesäniemi et al. 1987), as well as in adults with FH (Gylling et al. 1989a). In addition, serum plant sterol levels were higher in healthy children with apoE-4 phenotype compared with the children with apoE-3 phenotype (Tammi et al. 2001), hinting at more efficient cholesterol absorption in the children with apoE-4 phenotype. In contrast to these earlier studies, the sitosterol ratio to cholesterol was lowest in the non-FH children as well as in the combined group of FH and non-FH children with the apoE-4 phenotype (Study I). All the families in the present study had received dietary advice to control the children's dietary intake of saturated fat and cholesterol, whereas adult subjects from an earlier study were studied on their regular home diet (Kesäniemi et al. 1987). Thus, the discrepancy of the present results and results from adults could be explained by low intake of dietary cholesterol by the children of the present study, since there was no association between different apoE phenotypes and cholesterol absorption in study populations consuming a low cholesterol diet (Miettinen et al. 1992a, Bosner et al. 1999). However, the healthy 13-month old children from the study by Tammi et al. (2001) had received dietary recommendations, and their dietary intake of cholesterol was low. Thus, the discrepancy of the results from the children of the present and an earlier study (Tammi et al. 2001) remains a mystery.

Statin treatment has increased the serum plant sterol levels in short- (Uusitupa et al. 1992, Vanhanen et al. 1992, Vanhanen and Miettinen 1992a) and long-term (Miettinen et al. 2000a, Miettinen and Gylling 2003, Miettinen et al. 2003) studies. Thus, the statin-treated FH subjects in Study III in the beginning of the study had

higher plant sterol levels than the FH subjects of a similar age without lipid-lowering medication (e.g., campesterol 508±50 10^2 x mmol/mol of cholesterol vs. 187±20 10^2 x mmol/mol of cholesterol) (Gylling and Miettinen 1988). This increase of serum plant sterols has been assumed to be caused by decreased biliary excretion of plant sterols during statin treatment (Miettinen 1991). However, recently it was also found out that treatment with a high dose of atorvastatin in subjects with low baseline cholesterol absorption led to increased cholesterol absorption efficiency accompanied by increased ratios of plant sterols and cholestanol (Miettinen and Gylling 2003). The present study also showed that those subjects being given the higher dose of statin had higher baseline ratios of cholestanol to cholesterol, suggesting that their cholesterol absorption efficiency was higher. However, no direct conclusion from statin-induced increase in cholesterol absorption could be made, since the true baseline data prior the statin treatment were not obtained.

In the homozygous FH subject, the serum plant sterol and cholestanol ratios were four to nine times higher than in the heterozygous FH parents (Study IV). The surgical procedures as well as long-term statin and LDL apheresis treatments have apparently altered the sterol metabolism in the homozygous FH subject. However, some data exist from our homozygous FH subject gathered over the past few years. After the surgical procedures, but before the statin therapy was started, the levels of serum plant sterols were already high, and became further increased immediately after the start of statin treatment (Miettinen 1990). Thus, statin therapy apparently affects the serum plant sterol levels also in the homozygous FH subject, but the reason for the remarkably high serum plant sterol levels is possibly a sum of several factors. Due to the complicated cholesterol metabolism in the homozygous subject, no conclusions on cholesterol absorption efficiency compared with heterozygous parents can be made.

6.3.2. Effects of phytosterols

Serum cholesterol precursors

Based on cholesterol homeostasis, the inhibition of cholesterol absorption by dietary phytosterols leads to loss of sterols in feces, and this is followed by a compensatory increase in cholesterol synthesis (Gylling and Miettinen 1994b, Gylling and Miettinen 1996, Gylling et al. 1997, Jones et al. 2000). Since they are a marker of increased cholesterol synthesis, serum levels of cholesterol precursor sterols are increased by consumption of dietary phytosterols in adults (Hallikainen et al. 2000). In children with FH, the serum cholesterol precursor sterols have been evaluated only during consumption of plant stanol esters (Gylling et al. 1995, Vuorio et al. 2000). In accordance with these earlier studies, the ratios of cholesterol synthesis markers (mostly desmosterol and lathosterol) increased by as much as 36% with both spreads in hypercholesterolemic children (Study II). Thus, inhibited cholesterol absorption was partly compensated by increased cholesterol synthesis.

Despite the simultaneous inhibition of cholesterol synthesis by statins, serum cholesterol precursors were increased in adult FH subjects by both phytosterol esters (Study III). In particular, the ratio of lathosterol to cholesterol was increased by 15%

by both spreads. Previous studies conducted in statin-treated subjects did not show any significant increments in the serum lathosterol level during consumption of plant sterol ester spread (Neil et al. 2001, O'Neill 2003). However, studies in statin-treated subjects consuming plant stanol ester spread have reported conflicting results. Two studies showed 16-19% increments of serum lathosterol (Vuorio et al. 2000, Gylling and Miettinen 2002c), whereas no significant increments were seen in other studies (Vanhanen 1994, Gylling and Miettinen 1996, O'Neill 2003). The negative correlation between baseline levels and respective changes of most cholesterol synthesis precursors in the statin-treated FH adults (Study III) suggests that cholesterol synthesis is increased to the greatest extent in those subjects with the lowest baseline values. Statin treatment decreases the serum ratios of cholesterol precursors (Kempen et al. 1988, Uusitupa et al. 1992, Vanhanen et al. 1992, Vanhanen and Miettinen 1992a, Pfohl et al. 1998) and the decrease was greater with the more potent statin, atorvastatin, than with simvastatin (Miettinen et al. 2003). In some earlier studies conducted with a combination of statins and phytosterols (Vanhanen 1994, Gylling and Miettinen 1996, Vuorio et al. 2000, Gylling and Miettinen 2002c) lower doses of statins were used compared with the present study. In addition, in the study by O´Neill (2003), the baseline levels of lathosterol were three times higher than in the present study, but there is no data of which statins were used or at which doses. However, neither the present nor the earlier studies were designed to evaluate the effects of different statin doses during consumption of phytosterol esters. However, indirect conclusions from earlier studies with the data from the present study can be drawn that potency and dosage of statins affect first the baseline values of serum precursor sterols and subsequently the response of serum precursor sterols to phytosterol treatment.

Serum phytosterols and cholestanol

Serum cholestanol is a surrogate marker of cholesterol absorption efficiency (Miettinen et al. 1989), and serves as the only reliable marker of cholesterol absorption during consumption of the phytosterol ester products. Thus, serum ratios of cholestanol were decreased by both spreads in hypercholesterolemic children and in statin-treated FH subjects suggesting that cholesterol absorption had been inhibited by the phytosterol ester products. However, significant reductions were obtained only with the sterol ester spread. In fact, similar results were obtained also earlier in a comparison study with plant stanol and sterol ester spreads (Hallikainen et al. 2000). The fact that plant stanol esters resulted in similar serum total and LDL cholesterol reduction as the plant sterol ester spread, does not support the idea that cholesterol absorption would have been inhibited more by the sterol ester spread. Additionally, recent studies have reported similar, ~30%, reduction in cholesterol absorption by plant stanol and sterol esters (Jones et al. 2000, Normén et al. 2000). Thus, the reason for different response of serum cholestanol levels by the two phytosterol ester products remains unknown, but could be related to quantitatively small changes in serum cholestanol levels.

Plant sterol ester spread increased the serum campesterol and sitosterol levels in hypercholesterolemic children by 43-52% (Study II). The positive correlation

between the baseline sitosterol ratios with the respective changes suggests that in subjects with the highest sterol absorption, one observes the highest increase of serum sitosterol by dietary plant sterols. After consumption of 1.7 g of plant sterols for 5 weeks by the children in the present study, the highest concentration of plasma phytosterol, calculated as the sum of sitosterol and campesterol, was 3.5 mg/dl. This is far from the total plant sterol values of up to >25 mg/dl (mean 9 mg/dl) reported by Mellies and co-workers (1976a) in infants with even smaller doses of dietary plant sterols (300-400 mg/day). The reason for those remarkable elevations remains unknown. The increments of serum plant sterols observed in the current study did not differ from those reported in adults (Weststrate and Meijer 1998, Hallikainen et al. 2000). Accordingly, these results do not support the idea suggested by Mellies et al. (1976a) that the absorption of additional dietary plant sterols would be enhanced in children.

Since statin therapy increases the serum plant sterol levels, the baseline statin treatment may affect the changes of serum plant sterols during phytosterol ester consumption. However, only two studies have reported how the combination therapy with statins and plant sterol esters can affect the serum levels of plant sterols (Neil et al. 2001, O'Neill 2003). In the present study, serum campesterol and sitosterol ratios were increased by 74% and 48%, respectively by plant sterol esters in statin-treated FH subjects. These percentual increments are almost twice as high as have been reported in earlier studies (Neil et al. 2001, O'Neill 2003). The increase of serum plant sterols was highest in subjects with the highest respective baseline levels (Study III). However, in the study of O'Neill (2003), the baseline values of serum plant sterols were similar or even higher than in the present study, suggesting that the different baseline serum levels of plant sterols could not account for the greater increase of serum plant sterols caused by the sterol ester spread in the present study. One reason for the discrepancy in increments of serum plant sterols could be the different doses of dietary plant sterols consumed (1.4 g in the study by O´Neill vs. 2.0 g in the present study). In addition, the type of statin used and its dose, not mentioned in earlier studies (Neil et al. 2001, O'Neill 2003), affects the serum plant sterol levels. Accordingly, a more potent statin leads to a greater increase in serum plant sterol levels (Miettinen et al. 2003). Similar results were obtained also in the present study, since serum plant sterol ratios were increased most in those subjects receiving high-dose statin. Thus, the serum plant sterol levels in statin-treated subjects are increased by the sterol ester spread most in subjects with the highest serum baseline plant sterol values, this being partly attributable to the use of a more potent and higher dose of statin.

The mean concentration of total serum plant sterols in the heterozygous statin-treated FH subjects was ~2.5 mg/dl during consumption of the plant sterol ester spread, while in the two FH subjects without statin treatment, the total serum plant sterols amounted to ~1.6 mg/dl. Only two adult subjects without statin treatment could be studied, but this difference between the study groups gives some idea of the effect of statin treatment on serum plant sterol levels. Thus, prior statin therapy leads to even higher increments of serum plant sterols during consumption of plant sterol esters. Whether the increase of serum plant sterols of this magnitude is related to an

increased risk for CHD, is still unclear. However, in the homozygous subject, a major increase of serum concentrations of total plant sterols, up to 14 mg/dl, was obtained during consumption of plant sterol ester spread. This value approached the lower range of the serum plant sterols found in phytosterolemia (Björkhem et al. 2001), and thus could be considered as an additional risk factor for CHD.

Dietary plant stanol esters have reduced serum plant sterol levels in many earlier studies in adults (Vanhanen et al. 1993, Vanhanen et al. 1994, Gylling et al. 1999, Hallikainen et al. 2000) and children (Gylling et al. 1995, Tammi et al. 2000, Vuorio et al. 2000), as well as in combination with statins (Vanhanen 1994, Gylling and Miettinen 1996, Vuorio et al. 2000, Gylling and Miettinen 2002a, O'Neill 2003). In the present study, the plant stanol ester spread decreased the levels of serum plant sterols by 20-40% in all study groups. A negative correlation between the baseline absorption markers and their respective change in children suggests that the decrease of serum plant sterol values was highest in those subjects with the highest respective baseline values. However, this negative correlation suggests also that the reduction in serum plant sterols may remain undetectable in subjects with low baseline values. Since serum plant sterol and cholestanol values are positively related to the cholesterol absorption efficiency under steady state (Tilvis and Miettinen 1986, Miettinen et al. 1989, Miettinen et al. 1990b), the baseline serum plant sterol and cholestanol values apparently predict the baseline cholesterol absorption efficiency also in the present study. Thus, children with the highest baseline serum cholestanol and plant sterol levels have the highest baseline sterol absorption efficiency, and plant stanol esters inhibit the sterol absorption to the greatest extent in these subjects. The similar correlation shown in statin-treated FH subjects (Figure 9) suggests that plant stanol esters could eradicate the statin-induced increase of serum plant sterols from the highest but not from the lowest values of serum plant sterols. Thus, by combining plant stanols to statin therapy in FH, serum plant sterol levels approached the levels found in the normal population without statin treatment. In addition, in the homozygous subject, the serum plant sterol levels were decreased to even lower levels than obtained for several years previously without any statin treatment (Miettinen 1990). Even though animal studies have shown regression of atherosclerotic changes by feeding plant sterols (Ikeda et al. 1981, Moghadasian et al. 1997, Moghadasian et al. 1999a, Ntanios et al. 2003), recent indirect evidence suggests that increased serum plant sterol levels may be associated with CHD in humans (Glueck et al. 1991, Sutherland et al. 1998, Rajaratnam et al. 2000, Sudhop et al. 2002a, Assmann et al. 2003). Further data are needed to confirm the effects of serum plant sterol on development of atherosclerosis in non-phytosterolemic subjects. However, considering the possible role of high serum plant sterols as a risk factor for CHD, agents such as plant stanols, which reduce both LDL cholesterol and plant sterols in serum, could be recommended to be used alone or in combination with statins, especially for atherosclerosis-prone individuals, such as subjects with FH.

What is the role of increased serum plant stanols? Since plant stanols are absorbed in low but detectable amounts, serum plant stanol levels are increased by consumption of plant stanols (Gylling et al. 1999, Hallikainen et al. 2000), but interestingly, however, they were elevated also by consumption of a plant sterol ester spread in one

study (Neil et al. 2001). Even though the percentual increments of serum plant stanols are huge, the actual amounts of plant stanols remain very small, as also observed in the present study. In fact, the serum plant sterol levels during consumption of plant sterols were up to 40 times higher than the serum plant stanol levels during consumption of plant stanol esters. In animal studies, similar to the situation with plant sterols, plant stanol feeding led to a regression of atheromas (Ikeda et al. 1981, Ntanios et al. 1998, Volger et al. 2001). However, in stroke-prone spontaneously hypertensive rats, feeding a diet enriched with plant stanols lowered the survival rate compared with diet enriched with plant sterols (Ratnayake et al. 2003). These results could be related to genetic background of the study animals. In humans, no association between serum plant stanols and CHD has been found.

6.4. Distribution of non-cholesterol sterols in lipoproteins

The non-cholesterol sterol levels are normally measured from serum. Only a few studies have evaluated the non-cholesterol sterols in different lipoproteins. Therefore, we measured the non-cholesterol sterols from different lipoproteins in children with and without FH, as well as in the FH adults.

6.4.1. Baseline

In terms of total concentrations, LDL transported the major proportion of not only cholesterol but also of all the non-cholesterol sterols in the present study population (Studies I, III, and IV). In the homozygous FH subject with the highest serum cholesterol levels, LDL transported the highest percentage, ~90%, of non-cholesterol sterols (Study IV). This suggests that lipoproteins transport the non-cholesterol sterols in conjunction with cholesterol. The increased percentual proportions of all sterols in LDL in FH suggests that LDL is rich in all sterols to be carried to extrahepatic tissues. On the other hand, the decreased proportions of all the sterols in HDL in FH suggests that the reverse transport of sterols may be reduced when compared with normolipidemic subjects. Another explanation could be increased CETP activity in FH (Inazu et al. 1992), which would be accompanied by decreased sterol levels in HDL in the FH group. This increased CETP activity could increase the non-cholesterol sterol contents in VLDL and IDL. In fact, the concentrations of some non-cholesterol sterols were higher in VLDL and IDL of FH children compared with non-affected controls (Table 3, in Study I), even though the proportions of non-cholesterol sterols in TRL were lower in FH than in non-FH children (Figure 4).

A few earlier studies have reported high plant sterol ratios in HDL and high cholesterol precursor ratios in VLDL in different study populations (Tilvis and Miettinen 1986, Koivisto and Miettinen 1988a, Gylling and Miettinen 1994a, Miettinen and Gylling 2003). In the present study, the non-cholesterol sterol values in serum and different lipoproteins were further compared especially in Study I, when the medication as a confounding factor was avoided. This comparison was made in order to find out, whether any advantages in evaluating the cholesterol metabolism could be obtained by analyzing the non-cholesterol sterols in different lipoproteins. In fact, it was determined that the serum ratios of cholesterol precursors underestimate the respective levels in VLDL and overestimate those in HDL (except squalene). On

the other hand, serum ratios of the absorption marker sterols underestimate the respective levels in HDL, and overestimate those in VLDL. A similar distribution was obtained in the other study populations (Studies III and IV). Thus, neither statin nor apheresis treatments affect the distribution of non-cholesterol sterols in different lipoproteins. Accordingly, though it does involve time-consuming ultracentrifugation and additional GLC-runs, cholesterol synthesis or absorption or their changes can be detected better by measurement of non-cholesterol sterols in different lipoprotein fractions than in serum.

The present study confirmed that in terms of ratios to cholesterol, all the absorption markers are most abundant in HDL and less abundantly present in VLDL, whereas the ratios of cholesterol precursors are most abundant in VLDL. HDL is known to transport plant sterols from the tissues to bile, at least in phytosterolemia (Robins and Fasulo 1997), and LCAT, the enzyme partly responsible for the reverse cholesterol transport, is capable of esterifying also plant sterols (Nordby and Norum 1975). Thus, the high ratios of the plant sterols in HDL may reflect their reverse transport. The low levels of these sterols in VLDL could be attributable to the fact that plant sterols are not synthesized by the liver. It may also be speculated that dietary plant sterols and cholestanol are incorporated directly from CM to lipoproteins with higher density, such as LDL and HDL.

Endogenous hepatic cholesterol synthesis increases the hepatic amounts of synthesis markers, some of which are released in the blood circulation in VLDL. After lipolysis, VLDL is converted into IDL. This could be the explanation for the large amount of precursors, and especially squalene and lathosterol in VLDL and IDL (Study I). In addition to VLDL, CM was also abundant with cholesterol precursors for unknown reasons in all adult FH subjects. However, even though IDL is converted to LDL, the ratios of squalene and and lathosterol were low in LDL. The lower levels of cholesterol precursors in LDL could possibly be due to their rapid turnover rate, i.e., their half-lives have been shown to be only a few minutes (Miettinen 1970).

6.4.2. Effects of phytosterols

The concentrations of plant sterols were increased in all lipoproteins by the sterol ester spread. However, the percentual proportion of plant sterols carried by HDL was increased and that transported by LDL was decreased by both spreads in statin-treated FH subjects (Figure 10a). Similarly, the percentual proportion of plant sterols was decreased in LDL and increased in HDL by statin treatment (Miettinen and Gylling 2003). Thus, even though different changes occur in lipoprotein plant sterol concentrations during different treatment regimens, such as plant stanols, plant sterols, and statin therapy, their percentual proportion is decreased in LDL and increased in HDL, owing to the decrease occurring in LDL cholesterol levels. When adjusted to cholesterol, the plant sterol levels were increased similarly in all lipoproteins by the sterol ester spread, whereas the decrease of plant sterols was similar in all lipoproteins by the stanol ester spreads in FH subjects with and without statin treatment and in the homozygous FH subject. Accordingly, during consumption

of both spreads, all the absorption marker ratios were most abundant in HDL, with the lowest levels being seen in VLDL.

Cholesterol precursor sterols were increased only insignificantly in different lipoproteins in statin-treated FH subjects during consumption of dietary phytosterols. This could be related to the small sample size (n=6) studied and to the simultaneous statin therapy, which inhibits the possible increase in cholesterol synthesis. However, the homozygous FH subject behaved differently from the heterozygotes. Even though no changes occurred in serum cholesterol precursor sterols, the lathosterol ratio to cholesterol was increased significantly in VLDL by the stanol ester spread. Surprisingly, the lathosterol ratio was even decreased by the sterol ester spread. This decrease in lathosterol ratios could be accompanied by decreased bile acid synthesis, as reported earlier for the sterol ester spread (Weststrate et al. 1999, O'Neill et al. 2002), or the enhanced conversion of lathosterol to cholesterol for unknown reasons. Reduced cholesterol synthesis is not likely to have occurred, since cholesterol absorption was apparently still effectively inhibited.

6.5. Red cell sterols

Since increased sterol levels in red cells may cause episodes of hemolysis, we measured cholesterol and non-cholesterol sterols in red cells in hypercholesterolemic children and statin-treated FH adults at baseline and during consumption of phytosterol esters. The red cell offered also a model of a tissue cell in order to study simply the accumulation of different sterols in cells at baseline and during consumption of dietary phytosterol esters.

6.5.1. Baseline

The red cell membranes contain only free sterols, and only free sterols from serum are incorporated into the red cell membrane (Shohet 1972). In plasma, ~70% of cholesterol, desmosterol, and plant sterols and ~50% of lathosterol are in an esterified form (Salen et al. 1970, Gylling et al. 1993). Since LCAT is capable of esterifying also other sterols in serum (Nordby and Norum 1975), the activity of this enzyme regulates the levels of free sterols in serum, and indirectly influences also the levels of sterols in red cells. Thus, in spite of the fact that only ~30-50% of the sterols in serum are incorporated into the red cell membranes, the baseline data showed that the plant sterol, cholestanol, desmosterol, and lathosterol levels in red cells correlated positively with the respective values in serum (Studies II and III). This suggests that free non-cholesterol sterols from serum are incorporated freely into the red cell membranes, as shown also earlier for plant sterols (Ashworth and Green 1964, Salen et al. 1970).

6.5.2. Effects of phytosterols

Diet enriched with free sitosterol increased the plant sterol levels up to three times in red cells similarly as in plasma in one adult patient (Salen et al. 1970). In addition, the recent study by Hendriks et al. (2003) demonstrated that consumption of plant sterol esters for one year increased the plant sterol levels in red cells by ~80%. Thus, in

accordance with those earlier studies, the red cell plant sterols were increased by 40-75% during plant sterol ester consumption in the present study (Studies II and III). However, the total plant sterol values in red cells (~1.5 mg/dl) remained far from the values obtained in two phytosterolemic patients (12-13 mg/dl) (Bhattacharyya and Connor 1974). On the contrary, consumption of plant stanol esters decreased the contents of plant sterols in red cells, with the reduction being highest in those subjects with the highest baseline values. The respective increase and decrease of plant sterols in serum during consumption of plant sterol and stanol esters correlated with the similar changes in red cells (e.g., Figure 12a). Thus, when the serum levels of plant sterols are either increased or decreased, similar changes occurred also in red cells. This further suggests that plant sterols exchange freely between the lipoproteins and red cells. Similarly, cholesterol precursor sterol ratios were increased in red cells in a similar manner to the changes occurring in serum. However, the proportional changes of lathosterol ratio were not similar in serum and in red cells, as shown in Figure 12b. Accordingly, the increase of lathosterol was higher in red cells than the respective increase seen in serum. Similarly, it was earlier demonstrated that lathosterol was efficiently taken up by skin fibroblasts e.g., when compared with cholesterol (Leppimäki et al. 2000). This could be related to the lower esterification percentage of serum lathosterol compared with cholesterol and plant sterols. Thus, even as much as 50% of lathosterol, released by the liver as a consequence of increased cholesterol synthesis, remains in a free form and is available to be taken up by the red cells.

In contrast to the non-cholesterol sterols, the cholesterol concentrations in the red cells remained unchanged during both interventions (Studies II and III). As stated above, only free cholesterol is incorporated into red cell membranes. Thus, the reason for unchanged cholesterol levels in the red cells could be related to the fact that even though serum total cholesterol was decreased, the serum levels of free cholesterol remained unchanged during both interventions. Surprisingly, in contrast to the high respective correlations of plant sterols, the red cell and serum free cholesterol values were, however, not related to each other neither at baseline nor during consumption of the phytosterol esters. Accordingly, the exchange of plant sterols between serum and the red cells seemed to be more efficient than that of cholesterol. However, *in vitro* compared with plant sterols, plasma cholesterol exhibited the highest exchange with red cell cholesterol (Edwards and Green 1972, Child and Kuksis 1982, Child and Kuksis 1983). However, some non-cholesterol sterols appeared to replace cholesterol in the red cells (Bruckdorfer et al. 1969, Child and Kuksis 1982). Thus, large amounts of dietary plant sterols could probably replace cholesterol in red cells. This would apparently lead to higher ratios of plant sterols to cholesterol, and to less extensive elevations in cholesterol precursor sterols to cholesterol in red cells as shown in the present study. To conclude, during consumption of plant sterol ester spread, the total amount of sterols, other than cholesterol, is increased markedly in red cells. On the other hand, during consumption of the plant stanol ester spread, despite there being some increase of cholesterol precursor sterols and plant stanols, the total sterol levels in red cells are decreased.

There have been no systematic studies on the effects of large amounts of dietary plant sterols on human red cells. High contents of plant sterols in red cells result in

hemolysis, in at least phytosterolemic patients (Björkhem et al. 2001), and may lead to increased osmotic fragility (Bruckdorfer et al. 1969). Even though contradictory results from animal studies during phytosterol-enriched diet do exist (Moghadasian et al. 1999b, Ratnayake et al. 2000, Ratnayake et al. 2003), a recent study in humans showed that increased plant sterol levels in red cell membranes did not contribute to any changes in membrane rigidity (Hendriks et al. 2003). However, according to another recent study, free cholesterol transported by the red cells may destabilize the atheromatotic plaque and make it more vulnerable to rupture (Kolodgie et al. 2003). It is not known whether increased levels of plant sterols or other non-cholesterol sterols in red cell membranes may also have a harmful effect on plaque stability.

6.6. Removal of intravenous Intralipid

We studied the removal of intravenous Intralipid-squalene mixture, containing in addition to squalene as a marker of postprandial lipoproteins also cholesterol, plant sterols, and TG, during consumption of phytosterol ester spreads in order to evaluate whether cholesterol absorption inhibition could alter the removal of an intravenously given CM-like emulsion.

6.6.1. Baseline

It should be born in mind that at baseline the removal of Intralipid-squalene mixture was studied in subjects with FH treated for several years with statins. Accordingly, although the function of their LDL apoB receptors was impaired, it could have been improved by the long-term statin treatment (Brown and Goldstein 1986), as also shown in FH (Twickler et al. 2000). LDL apoB receptor takes up postprandial lipoproteins, but its role in the clearance of postprandial lipoproteins in FH is not totally understood, since controversial results exist from studies conducted in subjects with FH (Weintraub et al. 1987, Rubinsztein et al. 1990, Eriksson et al. 1991, Cabezas et al. 1998, Mamo et al. 1998, Twickler et al. 2000, Watts et al. 2001). Thus, at baseline the removal of postprandial lipoproteins could have been within the normal limits, since even normal clearance of postprandial lipoproteins has been reported in FH (Weintraub et al. 1987, Eriksson et al. 1991, Watts et al. 2001). The clearance of Intralipid-squalene mixture has been studied before in normolipidemic subjects (Relas et al. 2001b), and some comparison between those earlier results with the present results could be made. The clearance curves of squalene and plant sterols were mostly similar in the present study compared with the earlier study, but longer half-lives for CM squalene and especially for CM campesterol, measured from the absolute concentrations, were observed in the present study (93±12 and 170±37 minutes for squalene and campesterol, respectively) compared with previous data (74±8 and 37±5 minutes for squalene and campesterol, respectively) (Relas et al. 2001b). This difference could be due to different study populations, or could be related to different time points for the first measurements (10 minutes vs. 2.5 minutes), because the clearance of campesterol is fastest during the first ten minutes (Relas et al. 2001b).

6.6.2. Effects of phytosterols

Since plant stanols upregulate the expression of LDL apoB receptors (Plat and Mensink 2002b), and plant stanols improved the clearance of postprandial lipids in normolipidemic subjects (Relas et al. 2000), we expected that consumption of phytosterol esters would improve the clearance of TRL also in the present study. However, in this study, there was no improvement in the clearance of postprandial lipoproteins. Accordingly, TG removal from CM and VLDL was not affected during consumption of the dietary phytosterols, but CM squalene concentrations and the respective AUICs were unexpectedly even higher during consumption of the two spreads vs. baseline. In addition, the peak concentration of sitosterol was also higher during consumption of phytosterol esters vs. baseline. This indicates that the inhibition of cholesterol absorption and subsequent changes in cholesterol metabolism by phytosterols had no effect on TG removal from injected CM-like particles, but impaired especially the removal of CM squalene.

What is the reason for accumulation of squalene, but not TG, in CM-like particles after injection of intravenous Intralipid-squalene mixture? Statin treatment leads to a lack of liver cholesterol, and probably subsequently reduced VLDL synthesis (Thompson et al. 1996). Dietary phytosterols inhibit the intestinal absorption of cholesterol and thus prevent further entry of cholesterol into the liver. In fact, VLDL production was decreased in non-FH subjects during combination treatment with statins and plant stanols (Gylling and Miettinen 1996). Since CM and VLDL particles are taken up by the same hepatic receptors, the reduced release of VLDL could be associated with a rapid removal of injected TG during the first ten minutes when removal of squalene was less rapid. According to this theory, the removal of CM TG should have been improved during consumption of phytosterol esters. In fact, the peak concentrations of CM TG were lower during phytosterol ester spreads vs. baseline. However, the effect of cholesterol absorption inhibition by phytosterol esters was not sufficient enough to cause a significant difference in this small study population.

Thus, what could be the reason for the increased concentrations of TRL squalene during consumption of phytosterol esters vs. baseline? Sitosterol was found in addition to TRL, also in HDL and LDL particles after the intravenous injection (Relas et al. 2001b). Thus, sitosterol may be transferred rapidly to more dense particles, probably by lipid transfer proteins. However, since squalene was not present in LDL and HDL particles (Relas et al. 2001b), it must be assumed that squalene, as a nonpolar hydrocarbon, is not transferred between the lipoproteins. This suggests that dietary or intravenously given squalene is carried only by $d<1.006$ g/ml particles and taken up by the liver. This suggests that the impairment in removal of TRL squalene would be due to decreased uptake of TRL by the liver. The reason for this impaired removal during phytosterol ester consumption remains unknown, but could be related to this special study population. Thus, this suggests that in the present statin-treated FH subjects, dietary phytosterols did not upregulate the LDL apoB receptors, as seen earlier with plant stanol esters (Plat and Mensink 2002a). Accordingly, it seems that

the statin-treated FH subjects have only a limited capacity to upregulate their LDL apoB receptor activity during consumption of dietary phytosterols.

Consumption of plant stanol ester spread decreased the plant sterol concentrations in serum, CM, and VLDL, whereas plant sterol esters increased those levels. However, these changes did not affect the removal of postprandial plant sterols, since rather similar postprandial curves of plant sterols were obtained during consumption of both spreads. In a previous study, the immediate post-injection concentrations of plant sterols in CM were higher (Relas et al. 2001b) than the values obtained in the present study at 10 min (e.g., campesterol ~40 µg/dl vs. ~20 µg/dl). Accordingly, we speculate that immediate post-injection contents of CM plant sterols must have been higher in the present study during consumption of the sterol ester spread than with the stanol ester spread because of the higher pre-injection values. This indicates that the initial removal of plant sterols is rapid despite higher plant sterols concentrations if they are to reach similar incremental concentrations at 10 min and later. In fact, in the last measurements, the increments of plant sterols in VLDL were even lower during the plant sterol ester consumption compared with the respective increments during plant stanol ester consumption.

To conclude, consumption of dietary phytosterols impaired the removal of postprandial lipids in statin-treated FH subjects, when measured with CM squalene. The altered plant sterol concentrations of serum, CM, and VLDL did not affect the removal of plant sterols. The reasons for the results are not known, but could be associated with impaired removal of the lipids by the abnormal LDL apoB receptors present in FH subjects.

7. SUMMARY AND CONCLUSIONS

The results of the present thesis can be summarized as follows.

1. The concentrations of non-cholesterol sterols in serum and most lipoproteins were higher in FH than in non-FH children, whereas the serum and lipoprotein ratios of non-cholesterol sterols to cholesterol were similar in the two groups.

2. In all study populations, the ratios of cholesterol precursor sterols were higher in VLDL and those of absorption marker sterols were higher in HDL, when compared with the serum values. Even though analysis of non-cholesterol sterol ratios in serum provides a picture of cholesterol metabolism in general, the respective ratios in different lipoproteins may predict more exactly cholesterol metabolism and its changes.

3. Dietary plant sterols increased the serum, lipoprotein, and red cell plant sterol levels, whereas dietary plant stanol esters resulted in a decrease in these respective values. The highest increase of serum plant sterols was obtained in those subjects with the highest baseline levels of serum plant sterols. The changes of plant sterols and lathosterol in red cells correlated with the changes in serum. The plant sterol levels became changed similarly in all lipoproteins by the phytosterol ester spreads. Since recent evidence has suggested that high serum plant sterol levels may be associated with increased risk for CHD, the evidently increased serum plant sterol concentrations and ratios to cholesterol by consumption of plant sterol esters in hypercholesterolemic children and in statin-treated FH subjects, as well as in the homozygous FH subject may be considered as a possible additional risk for CHD. Based on the data from the literature, agents that lower both LDL cholesterol and serum plant sterols would be recommendable, especially in subjects with FH.

4. Dietary plant stanol and sterol esters were equally effective at decreasing LDL cholesterol in hypercholesterolemic children. In addition, dietary plant stanol and sterol esters reduced LDL cholesterol in statin-treated FH subjects. However, the results from this short-term study cannot predict the long-term benefit of dietary phytosterol esters.

5. Dietary phytosterols did not improve the clearance of postprandial lipoproteins in statin-treated FH subjects. In fact, the clearance of CM squalene, an indicator of postprandial lipoproteins, was even impaired during consumption of phytosterol ester spreads. The reason for impaired clearance of TRL remains unknown, but may be associated with altered sterol metabolism due to abnormal LDL apoB receptor function and the combination of statin and phytosterol ester treatment. Thus, since dietary plant stanols have improved the clearance of postprandial lipids in normolipidemic subjects in an earlier study, the present results could be attributable to this specific study population.

This thesis has increased our knowledge of the regulation of non-cholesterol sterols in serum, lipoproteins, and red cells in FH at baseline and during consumption of phytosterol esters. Special emphasis was paid to the levels of plant sterols in this study population, since awareness of the role of serum plant sterol levels as an additional risk factor for CHD has increased in recent years. However, the possible role of plant sterols in the development of atherosclerosis remains still open, and needs to be clarified in the future. In addition, the effects of plant stanol and sterol ester products as monotherapy or in combination with statins on development of atherosclerosis and incidence of CHD is still unknown and could be evaluated e.g., by measuring the intima-media thickness of the carotid artery or documenting the hard end points during long-term treatment.

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